Volume 16 JULY-AUGUST, 1951 Number 4

Food Research

An Official Publication of

The Institute of Food Technologists

Heureux déjà d'avoir pu servir mes concitoyens et l'humanite, je me repose sur la justice, la générosité et les lumières d'un gouvernement sagé qui ne cesse d'encourager et de protéger toutes les découvertes utiles.

—NICOLAS APPERT.

Published Six Times a Year in February, April, June, August, October, and December

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An Official Publication of
The Institute of Food Technologists

Volume 16 JULY-AUGUST, 1951 Number 4

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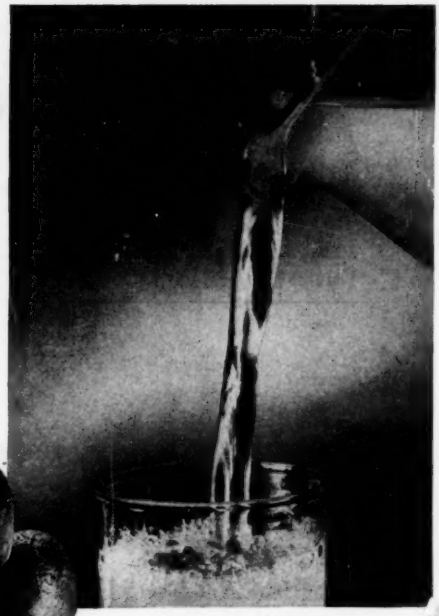

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EFFECT OF DIFFERENT ARTIFICIAL CASINGS ON THE MICROBIAL CHANGES IN REFRIGERATED LIVER SAUSAGE[a]

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(Manuscript received December 27, 1950)

It was shown recently that microorganisms can grow in refrigerated braunschweiger liver sausage stuffed in Visking and covered with Pliofilm *(2)*. The organisms grew most abundantly in the outer area of the sausage and it was concluded that they were stimulated by oxygen absorbed through the double casing. This work has now been extended to include other plastic films.

METHODS

Small liver sausages approximately 2.5 inches in diameter and 6 inches long were stuffed in different artificial casings. Methods of sampling and culturing were essentially the same as those already described *(2)*. Samples were taken from 3 areas or zones of each sausage, zone A being in the center, zone B midway between the center and the outside, and zone C next to the casing. Appropriate dilutions of all samples were cultured in: Difco tryptone glucose extract agar plates and Difco potato dextrose acid agar plates incubated aerobically at 30°C.(86°F.) for 5 days; BBL thioglycollate agar plates incubated anaerobically at 30°C.(86°F.) for 5 days; and Difco yeast dextrose agar shake tubes incubated anaerobically at 5°C.(41°F.) for 30 days.

Hydrogen ion concentration was determined by the method described by Watson *(3)* for Swiss cheese. Nitrogen was measured by the micro-Kjeldahl procedure of Johnson *(1)*.

RESULTS AND DISCUSSION

Effect of Casing on Changes in the Sausage. Portions of a single batch of liver sausage mix prepared in a packing plant were stuffed into the following casings: Pliofilm, 140 gauge; Pliofilm, 225 gauge; Saran, 200 gauge; and Visking. Pliofilm is made by the Goodyear Tire and Rubber Co., Akron, Ohio; Saran by the Dow Chemical Co., Midland, Michigan; and Visking by the Visking Corporation, Chicago, Illinois. All are commonly used packaging materials. Their physical properties can be learned from the manufacturers, but it might be said here that Visking is the most permeable to gases and water vapor while Saran is least permeable.

After the sausages were cooked and cooled, those in Visking were placed in individual envelopes of 140 gauge Pliofilm as is done commercially to prevent excessive dehydration. All sausages were stored in a household electric refrigerator at 5°C.(41°F.) and a single one taken from each lot at each of the 2 to 4 week sampling periods.

Table 1 shows bacteria counts and pH values for the outer zone (C) of each lot of sausage. The results for a single sausage chosen at random

[a] Published with permission of the Director of the Wisconsin Agricultural Experiment Station. This report is based on work supported in part by a grant from Oscar Mayer and Co., Madison, Wisconsin.

from the Visking plus Pliofilm group before storage were used as the initial figures for all lots. It is immediately apparent that considerable growth occurred in the sausages with the double casing, whereas there was none in those with a single casing. Also, the thickness of the Pliofilm (140 gauge vs. 225 gauge) had no effect. The significance of the apparent slight drop in pH of the Visking plus Pliofilm lot is doubtful because of the wide range of values obtained for individual sausages in which no growth occurred (see those in Saran and Pliofilm).

TABLE 1

Effect of casings on microbial and pH changes in the outer zone (C) of liver sausage stored at 5°C.(41°F.)

Casing	Days of storage	Counts per gram in[b]			pH
		TA	TGEA	YDA	
Visking plus Pliofilm (140 gauge)	0	170	5,200	<10	6.62
	15	6,500	12,000	5,700	6.48
	29	40	2,900	10	6.35
	50	250,000	570,000	1,000,000	6.32
	65	1,000,000	750,000	1,800,000	6.30
	78	1,800,000	2,400,000	2,600,000	6.38
Saran (200 gauge)	0	170	5,200	<10	6.62
	17	190	3,600	50	6.50
	31	80	1,600	<10	6.45
	52	160	3,200	100	6.50
	71	280	3,200	<10	6.51
	79	90	1,400	<10	6.54
Pliofilm (225 gauge)	0	170	5,200	<10	6.62
	18	250	1,600	10	6.42
	45	<10	420	10	6.40
	78	<10	60	<10	6.33
Pliofilm (140 gauge)	0	170	5,200	<10	6.62
	43	120	600	<10	6.41
	78	120	430	<10	6.41

[b]TA indicates thioglycollate agar; TGEA, tryptone glucose extract agar; and YDA, yeast dextrose agar.

The apparent decrease in numbers of aerobes (TGEA counts) in the sausages stuffed in Pliofilm is caused not by death of the organisms but by chance variation between sausages. Since the aerobic counts for the interior of these sausages were several thousands per gram, it must be assumed that the outer zones of the sausages sampled at 43, 45, and 78 days were heated more in cooking than were the others. Occasional variations in numbers of aerobic organisms in the outer zones of sausages from the same lot has been observed previously *(2)*.

The similarity of the counts in the 3 media for the sausages in Visking plus Pliofilm indicates the predominance of a single kind of organism that grew both aerobically and anaerobically at both 30°C.(86°F.) and 5°C. (41°F.). All of the organisms isolated from these cultures were gram positive, nonspore forming, catalase negative, microaerophilic rods that appeared identical to the unidentified organism described previously *(2)*.

The values for the A and B zones are not shown because they remained unchanged during storage. The figures for all lots were comparable to the results shown in Table 1 for the sausage in Saran. Also, the potato dextrose acid agar counts of yeasts are not tabulated because they remained at less than 10 per gram in all zones for the duration of the experiment.

The results in Table 1 raise the question of why organisms grew in the sausages with double casings but not in those with a single casing. In earlier work *(2)* the appearance of a greenish discoloration in the sausage next to the double casing was interpreted to mean that oxygen was absorbed through the coverings and thus stimulated growth of microaerophilic organisms in the outer zone. Results of the present study, however, indicate that this explanation for growth is inadequate. The greenish discoloration appeared not only in the sausages in Visking plus Pliofilm but also in those cased in Pliofilm alone, yet growth occurred only in the sausages with the double casing. That greening indicated oxygen absorption is shown by the fact that the sausage in Saran, which is less permeable to gases than are the other films, showed no change in color.

A possible explanation for the results described above may be found in the moisture content of the surface of the sausage. Water can be absorbed through Visking during cooking. Later when the outer casing is added the surface of the sausage is sprayed with water to facilitate its placement in the Pliofilm envelope. This operation traps small amounts of water between the casings and, because of the low permeability of the outer envelope, the water cannot evaporate readily. Thus the surface of the sausage is separated from a water film only by the readily permeable Visking. Perhaps dilution of the salt at the surface of the sausage adjacent to the casing enables organisms to grow there. There is no doubt that most of the growth occurs at the surface because when the casing is stripped back and an impression slide made from the surface, large numbers of organisms morphologically identical to those found in cultures can be seen.

In contrast with this, when liver sausage is stuffed and cooked in films much less permeable than Visking (Pliofilm or Saran), little or no water can be absorbed. Furthermore, the absence of an outer covering permits the surface to dry and thus there is no film of moisture in contact with the sausage to stimulate growth of organisms.

The final answer to the question of whether microorganisms grew in the Visking plus Pliofilm sausage because a double casing was used or because the casing next to the sausage was relatively permeable to water cannot be obtained from the results of this study. Perhaps if the number of layers in the casing were kept constant, for example by comparing Visking plus Pliofilm with Pliofilm plus Pliofilm, the question could be resolved. This has not been done because the intent was only to study casings as they are used in commercial practice. Unfortunately, as is often true, the results do not afford adequate answers to all the questions that arise.

It should not be assumed from the foregoing results that liver sausage stuffed in Visking and covered with a relatively impermeable casing invariably supports microbial growth. In one trial an experimental batch of

sausage in Visking plus Saran was stored for 64 days at 5°C.(41°F.) without showing growth. In this lot, however, the initial counts were only about 10 per gram, much lower than any observed in commercial sausage, and it must be assumed that if cold tolerant organisms were present in the original mix they were killed during cooking.

In all liver sausage with a double casing growth of organisms in the outer zone during refrigerated storage was accompanied by a greater or lesser increase in acidity (see also *2*). At the same time, however, other organisms grew as a slime between the casings and one could not be certain whether the observed decreases in pH were the result of acid formed by the organisms in the sausage or by those in the slime. Evidence favoring the latter is given by the experiment described above in which a heavy slime formed between the Visking and Saran casings, yet no growth occurred in the sausage itself. The pH of the outer zone decreased from about 6.4 to 5.4, thus indicating that the increase in acidity was caused by diffusion of acid from the slime through the Visking into the sausage.

Effect of Casing on "Slime" Development. The development of slime between the layers of double casings raises the question of whether slime formation was dependent on stuffing the sausage into a relatively permeable casing (e.g. Visking) or whether it was associated with the presence of an outer covering that prevented drying of the surface. To answer this question 2 lots of sausage were prepared, one stuffed in Visking and the other in Saran. Half of the Visking lot was covered with Saran and half of the Saran lot was covered with Visking. All were stored at 5°C.(41°F.) and examined at intervals for slime development.

Slime was visible in about 22 days on the sausages stuffed in Visking whether an outer casing was present or not. By contrast, sausages stuffed in Saran did not develop slime. As was expected, the sausages in Visking alone dried out considerably but the surface remained sufficiently moist to allow the slime to form. The organisms in the slime were mainly acid forming micrococci.

The above results indicate that slime forms on the surface of Visking because nutrients can diffuse out of the sausage, whereas it does not form on Saran because of the lesser permeability of the latter. A covering over the Visking no doubt improves conditions for slime formation by inhibiting evaporation of water.

To confirm the supposition that nutrients diffuse through Visking and thus promote slime development, samples of sausage stuffed in Visking and covered with Saran were stored at 5°C.(41°F.). At intervals a sausage was removed, samples of the liquid between the casings obtained with a small syringe, and the nitrogen content determined. Table 2 shows that

TABLE 2

Nitrogen content of the liquid between Visking and Saran casings

Days of storage	Mg. nitrogen per ml.	Days of storage	Mg. nitrogen per ml.
0	2.0	14	9.3
1	6.4	21	11.1
6	5.4	35	10.5
8	10.0		

the nitrogen content of the liquid increased rapidly for about 1 week, then apparently reached equilibrium and did not increase further. Slime was visible at 21 days. Thus it would appear that diffusion of nutrients through the Visking can account for growth of organisms between the casings.

SUMMARY

Liver sausage stuffed in Visking and covered with Pliofilm, then stored for several weeks at 5°C.(41°F.) permitted considerable growth of microorganisms in the area adjacent to the casing. There was no growth in sausage with a single casing of Pliofilm or Saran. The product in the double casing or in a single layer of Pliofilm developed a green discoloration after several weeks of storage; that in Saran retained its normal fresh appearance.

When liver sausage was stuffed in Visking and stored at 5°C.(41°F.) microbial growth developed on the casing. However, when it was stuffed in Saran no slime appeared. This result is believed to be caused by differences in permeability to nutrients of the two films. The organisms in the slime were predominantly acid forming micrococci.

Acknowledgment

We wish to thank Mr. Arthur Yaillen for his technical assistance in one of the experiments reported here.

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THE DIGESTIBILITY OF STEARYL ALCOHOL, ISOPROPYL CITRATES, AND STEARYL CITRATES, AND THE EFFECT OF THESE MATERIALS ON THE RATE AND DEGREE OF ABSORPTION OF MARGARINE FAT[a,b]

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(Manuscript received January 6, 1951)

The composition, functions, and non-toxicity of mixed isopropyl citrates, predominantly mono-isopropyl citrate (IC), and of the mixed stearyl citrates, predominantly distearyl citrate (SC), and of the alcohol components of these esters have been presented in an earlier publication *(3)*. Mention was made of the limited solubility of IC in oils requiring a vehicle for effecting dispersion of the IC in the oils. For this purpose a mixture of mono- and diglycerides was employed. This solution of IC will be referred to hereafter as IC plus Vehicle. The detailed composition of these additives to oils for purposes of flavor stabilization has been presented in the earlier paper by Deuel, Greenberg, Calbert, Baker, and Fisher *(3)*.

One purpose of the present study was to determine whether IC plus Vehicle or SC alters the rate of absorption of fat. Secondly, the digestibility of IC plus Vehicle and SC was also investigated. Finally, it was of importance to know whether either of the above components may exert any effect on the utilization of fat. In order to make a wider interpretation of the results possible, the digestibility studies were made on 2 species of animals, namely the rat and the dog. Tests were likewise carried out with a commercial stearyl alcohol to compare the utilization of this free alcohol with that of its citric acid esters.

EXPERIMENTAL

The studies on the effect of isopropyl and stearyl citrates on the rate of absorption of margarine fat were made by the procedure previously described *(4)*. Such an experimental approach would test whether IC plus Vehicle or SC exerts an inhibiting effect on the lipolytic enzymes or on other absorption processes.

The procedures usually employed in this laboratory *(2)*, modified as indicated below, were used for the digestibility tests. The experiments were made on 118 female rats, obtained from our stock colony, which weighed approximately 200 g. each. Ordinarily, 10 rats were used per group, although as few as 6 were employed in 2 cases, while one group consisted of 20 rats. The rats were placed on the diets containing the fats to be investigated for an orientation period of 5 days. Following this preliminary interval, the food consumption was recorded and the feces collected over a period of 8 days. The composition of the diets is recorded in Table 1.

The feces were analyzed for neutral fat and soap according to the procedures outlined by Augur, Rollman, and Deuel *(1)*. Any unabsorbed IC, SC, or stearyl alcohol would appear with the neutral fat and/or soap fraction.

[a] Carried out under a research grant from The Best Foods, Inc.

[b] Paper No. 276 from the Department of Biochemistry and Nutrition, University of Southern California.

The digestibility of the total fat in the diet in the tests with IC plus Vehicle (Groups 1 and 2), and of the margarine fat when fed alone (Groups 8 to 11), is calculated by the usual method *(1)*. In the case of the experiments on stearyl alcohol (Groups 3 and 4) and on SC (Groups 5 to 7), estimations of the digestibility of the stearyl alcohol or SC and of the margarine fat were calculated as indicated in the appendix.

The digestibility experiments on dogs were made over a 12-day period during the last third of the 12-week toxicological tests reported elsewhere *(3)*. The feces were separated by the use of carmine markers which were fed on the first and thirteenth days of the test. The feces were well formed and could readily be collected quantitatively. The methods for analysis are largely similar to those employed in the rat tests.

TABLE 1

Diets used in the digestibility experiments on rats

Diet and group number	Percentage composition							
	Casein	Margarine fat	Sucrose	Salt mixture[c]	Yeast[d]	IC plus Vehicle	Stearyl alcohol	SC
1	18.0	22.5	46.0	7.0	4.0	2.5	.....	.....
2	18.0	15.0	46.0	7.0	4.0	10.0	.....	.....
3	18.0	23.2	46.0	7.0	4.0	.....	1.8	.....
4	18.0	17.5	46.0	7.0	4.0	.....	7.5	.....
5	18.0	22.5	46.0	7.0	4.0	.....	.....	2.5
6	18.0	15.0	46.0	7.0	4.0	.....	.....	10.0
7	18.0	24.87	49.0	4.0	4.0	.....	.....	0.13
8[e]	18.0	15.0	56.0	7.0	4.0[f]	.....	.....	.....
9	18.0[g]	15.0	56.0	7.0	4.0	.....	.....	.....
10	18.0[g]	22.5	48.5	7.0	4.0	.....	.....	.....
11	18.0	25.0	49.0	4.0	4.0	.....	.....	.....
12	18.0	0	74.0	4.0	4.0	.....	.....	.....

[c] Osborne-Mendel salt mixture.
[d] Anheuser-Busch Strain G.
[e] Data from paper of Crockett and Deuel *(2)*.
[f] One per cent liver extract (Wilson, 1:20) replaced an equal proportion of yeast.
[g] Vitamin-test casein used. Commercial casein was employed in all other tests.

In order to determine whether the stearyl citrates could be hydrolyzed by the dog, samples of fresh feces were removed from the cage in the period directly after the completion of the digestibility tests. These were stored in the deep freeze until analyses could be completed. An aliquot was removed for the determination of the dried stool percentage. Another aliquot was used for the determination of any citric acid in an aqueous extract. Citric acid was also determined in the aqueous extract following saponification of another aliquot while an analysis for stearyl alcohol was made on the N.S.F. of this sample. The content of stearyl citrates in the stool was calculated on the basis that the citric acid makes up the same proportion of the ester mixture as in the original SC preparation fed. Any stearyl alcohol remaining over and above that accounted for in ester form was considered to be present as the free alcohol. The method of Pucher, Wakeman, and Vickery *(8)* as modified by Perlman, Lardy, and Johnson *(7)* was used for the estimation of citric acid.

Table 2 gives the composition of the diets used in the digestibility tests with dogs.

The isopropyl and stearyl citrates were obtained from The Best Foods, Inc., while stearyl alcohol was a commercial product purchased from du Pont which bears the trade name stenol. The identities of these products were established by analysis in our laboratory. The data on the citric acid esters are reported elsewhere *(3)*.

Stearyl alcohol was found to have the following constants: m.p., °C., 57 to 57.6 (59.4 to 59.8)[k]; and acid value, 0.0. Acetylated stearyl alcohol prepared from stenol gave the following values: saponification value, 177.6 (179.5, theory); m.p., 31 to 32°C. (32.85°C.)[k]; freezing point, 29.7°C. (30.25°C.)[k].

[k] The values in parentheses (except that marked theory) are from Heilbron *(6)*.

RESULTS

Experiments With Rats

Absorption Experiments. The absorption tests were made on male and female rats over 4- and 6-hour periods following the administration of margarine fat without or with IC plus Vehicle (3.33%) or SC (5%). The animals were adults which had been previously fasted for 48 hours. The margarine fat without or with the additives was fed at a level of approximately 300 mg./100 sq. cm. of surface area. A total of 91 rats was used in the study.

TABLE 2

Composition of the diets used in the digestibility tests with dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine fat[b]	18.3	18.24	15.3
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Cellu flour[1]	2.5	2.5	2.5
Salt mixture *(10)*	2.0	2.0	2.0
Dried brewer's yeast[1]	5.0	5.0	5.0
IC plus Vehicle	.....	0.06	.....
SC	.....	.....	3.0

[b] Without additives.
[1] Obtained from Chicago Dietetics Supply House.
[1] Anheuser-Busch, Strain G.

The rate of absorption was calculated by dividing the weight of the sample absorbed [weight of sample fed — weight of fat recovered from gut (corrected for residual content in fasting rat)] by the length of the experimental period. The correction for residual fat in the gastrointestinal tract of fasting male rats was found in these tests to be 59 mg. per rat while the figure obtained earlier for female rats which was used, was 30 mg. *(1)*. These values were employed for correcting for residual fat in the gastrointestinal tracts of the experimental animals which would not be of dietary origin.

A summary of the results of absorption is included in Table 3.

Neither IC nor SC would appear to have any depressing effect in rats on the rate of absorption of margarine fat from the gastrointestinal tract at the levels given. The concentration of IC plus Vehicle used in these tests is 133 times the amount at which it is proposed to use this mixture (0.025%) in margarine fat, while SC was incorporated at somewhat more than 25 times the proposed maximum for usage (0.15% in margarine).

Digestibility Experiments in Rats. Table 4 records digestibility experiments in which IC plus Vehicle, stearyl alcohol, or SC was added to the dietary fat at several levels. In all cases, sufficient margarine fat was included to bring the total level of fat plus test substance to 25% of the diet. The control experiments are summarized in Table 5. These include tests in which margarine fat was fed at 15, 22.5, or 25% of the diet without the above additives (Groups 8 to 11). Group 12 gives the experimental

TABLE 3

The absorption of margarine fat without or with IC plus Vehicle or SC from the gastrointestinal tracts of rats

Group no.	Category	Number of rats	Sex	Body weight after fasting	Fat			Fat recovered
					Fed	Absorbed		
						Total	Per 100 sq. cm. per hour	
	Margarine fat alone or containing 3.33% IC plus Vehicle							
				g.	*mg.*	*mg.*	*mg.*	%
1	Recovery							
	a. Margarine fat alone	8	M	280	1083	.....	.....	91.7
	b. Margarine fat with IC plus Vehicle	8	M	264	1044	.....	.....	89.5
2	Absorption (4 hours)							
	c. Margarine fat alone	10	M	265	1045	593	41.7	.....
	d. Margarine fat with IC plus Vehicle	9	M	259	1028	643	45.5	.....
3	Absorption (6 hours)							
	e. Margarine fat alone	9	M	266	1046	780	36.5	.....
	f. Margarine fat with IC plus Vehicle	9	M	283	1068	737	33.2	.....
	Margarine fat alone or containing SC (5%)							
4	Absorption (4 hours)							
	g. Margarine fat alone	10	F	158	766	352	43.2	.....
	h. Margarine fat with SC	8	F	158	797	345	43.5	.....
5	Absorption (6 hours)							
	i. Margarine fat alone	10	F	161	774	569	36.0	.....
	j. Margarine fat with SC	10	F	156	797	584	37.5	.....

TABLE 4

Summary of digestibility experiments on rats of fats containing IC plus Vehicle (Groups 1 and 2), stearyl alcohol (Groups 3 and 4), or SC (Groups 5 to 7)

Category	Group number						
	1	2	3	4	5	6	7
Number of rats used	9	9	10	9	9	10	9
IC plus Vehicle in diet, %	2.5	10.0	.....	.....	.....	.....	.....
Stenol (stearyl alcohol) in diet, %	.....	.....	1.8	7.5	.....	.....	.....
SC in diet, %	.....	.....	.....	.....	2.5	10.0	0.13
Increase in weight, g	4.1	5.2	3.2	−7.4	−2.0	0.0	−0.1
Eaten, fat, g	15.6	10.7	15.5	8.4	9.6	8.2	16.4
Eaten, test substance, g	1.74	7.16	1.20	3.58	1.07	5.45	0.086
Feces, dry weight, g	4.86	5.12	5.46	5.98	5.10	9.30	3.69
Feces fat, neutral fat, g	0.297	0.319	0.516	2.807	1.257	3.195	0.613
Feces fat, soaps, g	0.589	0.455	1.014	0.754	2.299	3.787	0.664
Feces fat, corrected N.S.F., g	.....	.....	0.131	1.632	0.902	3.419	0.032
Feces fat, total, g	0.886	0.774	1.530	3.561	3.556	6.982	1.277
Feces fat, corrected total,[1] g	0.638	0.515	1.254	3.258	3.357	6.734	1.035
Digestibility of fat alone[m]	96.2 ± 0.4	97.1 ± 0.3	91.9 ± 1.0	80.8 ± 2.1	77.1 ± 1.3	71.6 ± 3.0	94.1 ± 0.6
Digestibility of test substance[m]	.....	.....	88.6 ± 4.2	54.8 ± 5.7	5.7 ± 3.9	19.3 ± 4.8	54.3 ± 8.1

[1] Corrected for metabolic fat 50.5 mg. per g. dried feces. Group 7 is based on metabolic fat results of Group 12.

[m] Including the standard error of the mean calculated from the formula, $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$, where d is the deviation from the mean and n is the number of observations.

data of the tests for the determination of metabolic fat where a fat-free diet was employed. Ordinarily 10 rats were used per group although as few as 6 were employed in 2 cases, while one group consisted of 20 rats. All tests were made on female rats which weighed approximately 200 g. each since these were available at the time of the tests. A total of 115 animals was used in these tests.

TABLE 5

Summary of control digestibility experiments without fat or with margarine fat at several levels

Category	Group number				
	8	9	10	11	12
Number of rats used	20	6	6	10	8
Fat in diet, %	15.0	15.0	22.5	25.0	0
Increase in weight, g.	−1.0	2.8	2.5	−0.2	4.9
Fat eaten, g.	10.7	10.2	13.6	15.0	0.0
Feces, dry weight, g.	4.40	3.33	3.22	3.26	2.82
Feces fat, neutral fat, g.	0.240	0.113	0.342	0.468	0.107
Feces fat, soaps, g.	0.300	0.341	0.334	0.484	0.081
Feces fat, total N.S.F., g.	0.091	0.058	0.192	0.116	.....
Feces fat, total, g.	0.540	0.474	0.678	0.952	0.188
Feces fat, corrected total, g.[a]	0.310	0.306	0.516	0.750	0.066[c]
Coefficient of digestibility[b]	97.0 ± 0.4	97.0 ± 0.9	96.3 ± 1.5	95.2 ± 0.9	.....

[a] Corrected for metabolic fat of 50.5 mg. per g. dried feces. Groups 7 and 11 corrections are based on metabolic fat determined in Group 12.

[c] Total g. feces fat per g. dried feces (metabolic fat).

[b] Including the standard error of the mean calculated by the formula given in footnote [m], Table 4.

Experiments With Dogs

Digestibility experiments were made with 3 groups of 3 dogs each. One group comprised the controls in which the fat with no additives was fed, the second consisted of those in which the test diet included IC plus Vehicle (0.06% of the diet, 0.25% of the fat), while the final group received 3% SC in the diet (14.8% of the fat). The data of the individual tests are summarized in Table 6.

When the diet contained 18.3% margarine fat and 5% cod-liver oil without additives, the average coefficient of digestibility without correcting for the metabolic fat was 95.3. After correcting for the metabolic fat using the correction figure of 0.198 g./g. dried stool (5), the digestibility values for fat averaged 99.7%. The corresponding figures for digestibility of margarine fat containing IC plus Vehicle are 95.5% and 99.7% without and with the correction for metabolic fat.

On the other hand, considerably lower values were obtained for the digestibility of the lipid as a whole in the tests where SC was given. This average value was 79.8%. However, when correction is made for the SC and stearyl alcohol content of the feces, the coefficient of digestibility of fat (still uncorrected for metabolic fat) is approximately 87%. Finally, when the correction for metabolic fat is likewise applied, the mean coefficient of digestibility averages 94.5.

There are several assumptions that it has been necessary to make in order to calculate the digestibility of fat in these tests. In the first place,

TABLE 6

Digestibility tests of margarine fat and cod-liver oil in dogs when fed alone in the diet or with IC plus Vehicle or with SC

Category	Control tests			IC plus Vehicle tests			SC tests		
	Dog 1	Dog 2	Dog 3	Dog 5	Dog 6	Dog 7	Dog 9	Dog 10	Dog 11
Food eaten, g.	980	980	3962	974	974	3325	968	968	2870
Fat eaten, g.	228.3	228.3	923.1	226.9	226.9	775.0	196.5	196.5	582.6
Test substance eaten, g.	.....	.....	.....	0.58	0.58	2.00	29.1	29.1	86.1
Feces									
Dry weight, g.	49.1	68.7	284.7	52.3	43.7	207.5	86.8	103.7	221.6
Corrected dry weight, g.[q]	.....	.....	.....	.....	.....	.....	72.4	87.7	187.4
Lipid excreted									
Neutral fat, g.	4.40	4.59	36.9	6.71	3.65	22.8	26.7	35.8	69.1
Soaps, g.	3.60	3.35	29.3	2.70	3.05	26.2	10.5	12.1	30.1
Total, g.	8.00	7.94	66.2	9.41	6.70	49.0	37.2	47.9	99.2
Corrected for metabolic fat, g.[r]	0.00	0.00	9.2	0.00	0.00	8.0	.....	.....	.....
Corrected for SC and stearyl alcohol only, g.[q]	.....	.....	.....	.....	.....	.....	22.8	31.9	65.0
Corrected for SC, stearyl alcohol, and metabolic fat, g.	.....	.....	.....	.....	.....	.....	8.5	14.5	27.8
Coefficients of digestibility									
Total lipid	96.5	96.7	92.8	95.7	97.1	93.8	81.1	75.6	82.8
Fat									
Corrected for metabolic fat	100.0	100.0	99.2	100.0	100.0	99.0	.....	.....	.....
Corrected for SC and stearyl alcohol only	.....	.....	.....	.....	.....	.....	88.3	83.8	88.9
Corrected for SC, stearyl alcohol, and metabolic fat	.....	.....	.....	.....	.....	.....	95.6	92.6	95.2
SC alone	.....	.....	.....	.....	.....	.....	50.5	45.0	60.2

[q] Corrected by subtracting weight of SC plus stearyl alcohol (Item *o*, Table 7) from total weight of dried feces. [r] Corrected for metabolic fat by multiplying corrected dried feces weight by 0.198 *(5)*.

since no figure for estimating metabolic fat has been reported for the dog, the same value has been assumed as for man. This figure would appear to be too high, since the metabolic fat calculated from it exceeds the figure for total fecal lipid found in 4 of the 6 tests on fat alone or fat with IC plus Vehicle. Moreover, since some of the citric acid from the stearyl citrate esters in the stool may be unavoidably destroyed on saponification, the resulting calculations for non-fat residues in the feces may be too low. This would cause the calculated coefficients of digestibility of fat to be too low. Such an error would have only a small effect on the results (less than 1%).

TABLE 7

The determination of the proportion of SC and stearyl alcohol excreted in the feces of dogs after the administration of SC

Category	Dog 9	Dog 10	Dog 11	Dog 12
Stearyl alcohol excreted:				
(a) N.S.F., total, mg.	258(1)	219(4)	.....	230(2)
(b) N.S.F., control value, mg.	33	33	.....	33
(c) Stearyl alcohol, total, mg. (a-b)	225	186	.....	197
Citric acid excreted:				
(d) As free acid (before hydrolysis), mg.	0	0	.....	0
(e) Combined (after hydrolysis), mg.	46(1)	49(4)	.....	50(2)
Distribution of stearyl alcohol:				
(f) Total, mg. (c)	225	186	.....	197
(g) Combined,mg. (calculated from e)[a]	129	138	.....	140
(h) Free, mg. (f-g)	96	48	.....	57
Calculation of SC hydrolyzed:				
(i) Total stearyl alcohol as SC, mg. (calculated from c)[b]	290	240	.....	254
(j) Unhydrolyzed SC, mg. (calculated from e)[c]	166	178	.....	181
(k) Hydrolyzed, mg. (i—j)	124	62	.....	73
(l) Hydrolyzed, % (k/i × 100)	42.8	25.8	.....	28.7
Calculation of SC and stearyl alcohol content of feces in digestibility tests (Table 6):				
(m) N.S.F., total, gm.[v]	14.7	16.1	34.8	.....
(n) N.S.F., corrected, gm.[w]	12.3	13.2	28.6	.....
(o) Stearyl alcohol and SC in feces, g. $\left[(n \times l) + (100 - l) \times n \times \frac{696}{540}\right]$	14.4	16.0	34.2	.....

Figures in parentheses give number of determinations of which preceding value is the average. Values a-k represent mg. per g. of dried stool.

[a] Combined citric acid × 2.81 (540/192).

[b] Corrected stearyl alcohol × 1.29 (696/540).

[c] Combined citric acid × 3.63 (696/192).

[v] Value analytically determined.

[w] Control N.S.F. (estimated as 17.2% of dried feces) subtracted from *m*. Item *o* determined as indicated from tentative value of *n*. Value obtained in item *o* subtracted from weight of dried feces in Table 6. Corrected dry weight × 33 mg. gives the final correction to be applied to item *m* to give item *n*. Final calculation was made of item *o* by procedure indicated and this figure was used for the several computations in Table 6.

However, if citrate is destroyed on saponification, a marked error in the amount of free stearyl alcohol calculated will result. This would give a figure somewhat too high for the digestibility of SC itself. Moreover, a further assumption has been made that the citrate obtained after hydrolysis is combined with stearyl alcohol in the same proportions as it is in SC. If it were exclusively combined as tristearyl citrate, then the percentage

of free stearyl alcohol calculated would be too high; if it were combined largely as monostearyl citrate, then the reverse would be true. Such variations would not change the corrections made for calculations of fat digestibility.

DISCUSSION

The experiments reported here with rats indicate that IC plus Vehicle is completely digested when administered in amounts as high as 10% of the total diet or 67% of the fat. There is no evidence that the inclusion of such high proportions of this product in the food results in a lowering of the coefficient of digestibility of the fat included in the diet. Thus, the average figures for digestibility of the fat as a whole were 96.2 and 97.1 respectively for the margarine fat in rations containing 2.5 or 10% of IC plus Vehicle. The values for the digestibility of margarine without IC plus Vehicle when fed at a 15% level were 97.0% (2 series of tests); when fed at 22.5 or 25% of the diet, the average figures for digestibility were 96.3 and 95.2% respectively. The almost complete digestibility of margarine fat containing IC plus Vehicle is in line with the results of the tests on the rate of absorption. No essential differences were found in this index, irrespective of whether IC plus Vehicle was added to the diet or whether margarine fat without additives was used.

The results on rats with IC plus Vehicle were likewise duplicated on dogs. Thus, a mixture of margarine fat and cod-liver oil without or with IC plus Vehicle gave the following coefficients of digestibility: No correction for metabolic fat, 95.3, 95.5; corrected for metabolic fat, 99.7, 99.7.

On the other hand, the addition of stearyl alcohol to the diet of rats to the extent of 1.8% (7.8% on the fat) significantly lowered the digestibility of margarine fat to 91.1%. When 7.5% of stearyl alcohol was included in the diet (43% on the fat), the coefficient of digestibility of the fat was lowered to about 81, while that of stearyl alcohol itself was only 55.

Our experiments indicate that SC is poorly absorbed by the rat. When SC was added to the diets at levels of 2.5 or 10% (11 or 67% respectively on the fat), the calculated digestibility of the ester was 5.7 and 19.3% respectively. A concomitant reduction in the digestibility of margarine fat from the usual high level of 96 to 97% to approximately 75 per cent was noted. On the other hand, when SC was added to the diet in an amount of 0.13% (0.5% on the fat), which is about 3 times the maximum amount required to protect the fat, there was no appreciable lowering in the coefficient of digestibility of the margarine fat included in the diet (94.1).

The depression in absorption of margarine fat as the result of the enormous amounts of stearyl alcohol or SC added to the fat is attributed in part to the influence that these additives have on the melting point of fat so supplemented. Thus, a mixture of 10 parts of SC and 15 parts of margarine fat has a melting point of 123°F. (Wiley) compared to 94°F. for the margarine fat alone.[x] It has been repeatedly demonstrated that high-melting fats are poorly digested *(1, 2)*. Of interest in this same con-

[x] Data supplied by F. H. Luckmann, The Best Foods, Inc.

nection is the fact that when SC was fed to rats at a lower level (5% of the fat) but still very much in excess of that proposed for the use of the esters in fats, no interference in the rate of absorption obtains.

When a comparatively high amount of SC was introduced into the diet of the dog (3% of the diet or 15% on the fat), the digestibility of the fat was 94.5% although the SC itself was digested only about 52%. The fact that SC exhibits less inhibition on the digestibility of margarine fat in the dog than in the rat may be an example of species difference.

The data indicate that stearyl alcohol is largely absorbed in the rat when present at 1.8% of the diet (coefficient of digestibility = 89). This is in harmony with the results of Stetten and Schoenheimer *(9)*, who report a ready absorption of stearyl acetate when fed at a 1% level in the diet of normal rats. However, stearyl alcohol is less completely utilized when present in the diet at a 7.5% level (coefficient of digestibility = 55).

In view of the fact that stearyl alcohol is well absorbed at a 1.8% level, while SC is largely unabsorbed when fed at an equivalent concentration in the diet (2.5%), one is forced to postulate that the rat has a limited ability to hydrolyze this ester. On the other hand, there would seem to be ample evidence that the dog is able to split SC to a considerable extent. This conclusion is based on the fact that definite amounts of free stearyl alcohol have been demonstrated in the feces of the dog. Moreover, the calculated coefficients of digestibility of SC in the dog at a 3% level in the diet approximate 50, while in the rat the figure was 6 when the esters were present to the extent of 2.5% of the diet.

SUMMARY AND CONCLUSIONS

In an investigation of new additives for food usage, it is essential to supplement toxicological studies with biochemical investigations to determine the metabolic fate of the proposed food additives. This type of experiment is useful in explaining toxicological manifestations, if such should be noted.

Experiments have been carried out to determine the digestibility of stearyl alcohol, isopropyl citrates, and stearyl citrates, and the effect of these materials on the rate and degree of absorption of margarine fat. The latter is a typical food in which the citrate esters might be included. Incorporation of isopropyl citrate esters, predominantly the mono-isopropyl citrate in its mono- and diglyceride vehicle (IC plus Vehicle) in amounts as high as 10% of the diet results in a practically complete absorption of the product without lowering the digestibility of the dietary fat. Stearyl alcohol, included in this study for purposes of better interpretation of the experimental findings, is absorbed to the extent of 89% when given at a level of 1.8% of the diet. Under these conditions, practically no adverse effect on the digestibility of the fat given concomitantly is noted. However, when added to the diet to the extent of 7.5% (43% on the fat), stearyl alcohol is absorbed in the amount of 55% and the fat absorption is decreased to 81%.

Stearyl citrate esters, predominantly distearyl citrate (SC), are poorly absorbed by the rat when fed at 2.5 or 10% of the ration (11 or 67% respectively on the fat). The coefficient of digestibility of the fat fed with

SC is lowered from the usual 96 to 97% to 75%. However, when SC comprises 5% of the fat, no interference in rate and degree of fat absorption occurs. It would appear that the incomplete digestion of SC by the rat is due to the inefficient hydrolysis of these esters in the gastrointestinal tract.

The dog is able to digest SC more effectively than is the rat. Thus, at a level of 3% in the ration (approximately 15% on the fat), fully 50% is digested, and no significant interference with the absorption of margarine fat occurs.

It may be concluded that IC plus Vehicle fed at a level more than 2500 times that proposed for use under practical conditions, or IC alone in an amount about 1000 times the maximal proposed level, will in no way interfere with the digestibility of fat. In the case of SC, there is no significant interference in the absorption of fat when the SC is present in an amount somewhat more than 25 times the maximal proposed concentration. The interefrence in fat absorption at higher levels is attributed in part to the increase in melting point of the fat plus additive blend, an artificial situation created in an attempt to determine levels at which interference might be noted.

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APPENDIX

Methods of Calculation of Digestibility in Tests with Stearyl Alcohol (Stenol), Groups 3 and 4, in Rats

A. Calculation of digestibility of total fat by usual methods *(1)*.

B. Calculation of digestibility of fat alone.

a. Determination of total fat fed.

b. Total fat excreted (corrected) = Total fecal lipids — [Metabolic fat[a] + N.S.F. (corrected)[b]].

c. Digestibility of fat alone $= \frac{a - b}{a} \times 100.$

C. Calculation of digestibility of stenol.

d. Determination of total stearyl alcohol fed.

e. Stearyl alcohol excreted = Total N.S.F. excreted — Control N.S.F.[c] excreted.

f. Digestibility of stearyl alcohol $= \frac{d - e}{d} \times 100.$

Methods of Calculation of Digestibility in Tests With SC (Groups 5, 6, and 7), in Rats

D. Calculation of digestibility of total fat by usual methods *(1)*.

E. Calculation of digestibility of fat alone.

g. Determination of total fat fed.

h. Total fat excreted (corrected) = Total fecal lipids —

$$\left[\text{Metabolic fat}^{a} + \frac{\text{N.S.F.(corrected)}^{b}}{0.776^{d}}\right]$$

i. Digestibility of fat alone $= \frac{g - h}{g} \times 100.$

F. Calculation of digestibility of SC.

j. Determination of total SC fed.

k. SC excreted $= \frac{\text{Total N.S.F. excreted} - \text{Control N.S.F. excreted}}{0.776^{d}}.$

l. Digestibility of SC $= \frac{j - k}{j} \times 100.$

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[a] Metabolic fat is determined by total lipid excretion (neutral fat plus soap) on fat-free diet.

[b] Non-saponifiable fraction (corrected) = Total fecal N.S.F. — N.S.F. of control groups receiving 15 or 22.5% of margarine fat alone (Groups 9 and 10).

[c] N.S.F. of control groups (Groups 9 and 10).

[d] Factor for conversion of stearyl alcohol to equivalent amount of SC.

THE OXALIC ACID CONTENT OF SOME COMMON FOODS

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The significance of the oxalate content of foods has been emphasized by Fincke and Sherman *(4)* and by Fairbanks and Mitchell *(3)*. While for any given food the ratio of calcium to oxalate is the vital consideration *(7)*, a mixture of foods is usually undergoing digestion at any one time and the total oxalate in relation to the total calcium must be considered. The rachitogenic effect of excessive amounts of soluble oxalates in diets marginal with respect to calcium has been generally accepted. In such cases the calcium may be performing the desirable function of detoxifying the oxalates by precipitating them at the expense of its own absorption, possibly a beneficial effect if the diet contains ample calcium. However, ease of absorption of any excess of soluble oxalates and the resulting oxaluria frequently result in formation of kidney and bladder calculi. Approximately one-third of the latter are composed of fairly pure calcium oxalate; half or more contain at least some.

All the methods used for the determination of oxalates consist essentially of extraction of the oxalic acid from the food by means of acid with subsequent precipitation as calcium oxalate. The latter, dissolved in acid, is then titrated. Various modifications and refinements have been suggested but the basis of the method has almost invariably been that described above. In this connection, the following reports may be cited: Gregoire and Carpiaux *(5)*, Arbenz *(1)*, and Pucher, Vickery, and Wakeman *(9, 10)*.

Kohman *(6)* published the results of his estimation of the oxalic acid content of a large number of fruits and vegetables. His method consisted of extraction of the food sample at room temperature for two days with 15% hydrochloric acid. The resulting solution was then extracted with ether to remove the oxalic acid, the ether allowed to evaporate, and the oxalic acid precipitated as the calcium salt. The latter was then dissolved in sulfuric acid and titrated with standard permanganate in the usual way. Perhaps the most distinctive feature of Kohman's method is the determination of chloride in both the liquid and the solid portions of the food sample which had previously stood in contact with 15% hydrochloric acid for at least two days. Having separated the solid and the liquid portions of this digest by pressing through cheesecloth, Kohman assumed that any oxalic acid present would distribute itself between these portions in the same ratio as the chloride. The latter was determined by titration (presumably by the Volhard method) of a diluted sample of the filtrate. The determination of chloride in the solid residue is not described nor are any figures submitted to show the data obtained in a typical determination. Although Kohman *(6)* found that oxalic acid added to food samples distributed itself between the solid and the liquid phases in the same ratio as did the added chloride, we question whether a similar pro-

portional distribution can be safely assumed for the oxalic acid produced in the growing plant and no doubt much more thoroughly incorporated in its tissues. We have preferred the greater simplicity of exhaustive Soxhlet extraction of a sample of the food in a coarse sintered glass crucible by means of hydrochloric acid. As indicated below, this procedure gave quantitative recoveries of oxalic acid added to the food sample. While it may be argued that such added acid would not be incorporated into the interior of plant fibres, the same can be said for the added hydrochloric acid, on the distribution of which Kohman depended for his calculation of the oxalic acid content of the whole sample. Comparison of Kohman's method *(6)* with ours on the same foods has given similar results but with much greater variation between duplicate determinations. This is almost entirely because of difficulty in obtaining satisfactory duplicate determinations of the chloride content of the solid residue. In the interest of greater simplicity and speed we have therefore initiated the studies described below.

EXPERIMENTAL

Studies of the Method. Our method consisted, briefly, in extracting a 2- to 4-g. sample of the dried, finely ground food with hydrochloric acid in a Soxhlet extractor, extracting the resulting acid extract with ether, evaporating the ether, precipitating the oxalic acid as calcium oxalate, dissolving the precipitate in acid, and titrating with standard potassium permanganate. To establish optimal conditions for both the acid and the ether extractions, the optimal acidity and time for each was studied.

Of these four variables, the conditions for three were arbitrarily set while the fourth was varied. A large lot of spinach was dried in air at room temperature and then in an oven at 80°C. to constant weight. It was then ground to pass a 40-mesh sieve. The product was kept in a desiccator over phosphorus pentoxide.

A study was made of the optimal concentration of hydrochloric acid in the acid extraction using, for the other conditions, a 15-hour period of acid extraction, followed by ether extraction for 25 hours at unit normality of acid. That a 15-hour acid extraction was not optimal was shown later. In these acid extractions, 75 ml. of the acid was placed in the 125-ml. flask of the Soxhlet extractor and the weighed sample of the dried and ground food (2 to 4 g.) was placed in a coarse, sintered glass crucible which fitted easily into the extraction chamber. Syphoning of the extractor required a volume of about 50 ml. of the distilled acid. Thus the extent of distillation of the original 75 ml. of acid used precluded the possibility that any appreciable amount of acid would reach the sample unless the concentration of that remaining in the flask approximated the constant boiling solution of about 5.5*N*. In actual practice it was found that maximal values were attained with normalities of acid from 3 to nearly 7 as indicated in Table 1. Titration of samples of the distilled acid resulting from 2.89*N* HCl gave, before syphoning, a normality of about 0.2, showing that the volume in the flask had been reduced to the point of distilling over a small amount of the constant boiling acid. This was no doubt due to the amount of distillate in the condenser and side arms of the extractor, in addition to that accumulating around the food sample. Variations in the

TABLE 1

Effect of variation in the concentration of extracting hydrochloric acid upon the percentage of oxalic acid in spinach

Hydrochloric acid	Oxalic acid	Hydrochloric acid	Oxalic acid
N	%	*N*	%
0.10	0.25	3.48	0.96
1.05	0.49	4.16	0.94
1.68	0.72	4.34	1.00
2.89	0.94	6.78	0.97

ratio of volume of acid used to volume of the extractor will, of course, vary the minimal normality of acid to be recommended, but the use of from 3 to 5*N* hydrochloric acid appears to be a safe recommendation for most Soxhlet extractors. Although very water soluble, it is obvious that oxalic acid is not released from plant tissues by water extraction alone. Partial hydrolysis and softening of the cellulose by means of the acid is evidently essential to complete recovery.

Keeping the conditions for ether extraction the same as those described above, the effect of length of time of acid extraction was varied from 3 to 25 hours, using 3 different concentrations of acid within the range suggested above. Table 2 shows the

TABLE 2

Effect of variation of the time of acid extraction and the concentration of extracting hydrochloric acid upon the percentage of oxalic acid in spinach

Time of extraction, hours	Percent oxalic acid found when extracted with		
	2.89*N* HCl	3.48*N* HCl	4.16*N* HCl
3	1.05	1.05	1.04
5	1.04	1.06	1.04
10	1.00	1.01	1.01
15	0.94	0.96	0.94
20	0.93	0.94	0.96
25	0.93	0.95	0.94

data obtained on the same lot of ground and dried spinach, expressed in terms of the percentage anhydrous oxalic acid in the fresh plant. It is obvious that the 15-hour extraction period is not optimal and that extractions longer than from 3 to 5 hours appear to result in some decomposition, a finding verified by experiments with pure oxalic acid. There seems to be little choice between the three acid normalities employed.

The effect of the acidity of the acid extract on the recovery of oxalic acid by ether extraction, for 25 hours from a series of solutions 0.005*M* with respect to oxalic acid, is shown by the data in Table 3.

TABLE 3

Effect of varying the acidity of the oxalic acid-hydrochloric acid mixture before ether extraction upon the percentage recovery of 0.05*M* oxalic acid

Added HCl, *N*	Recovery, %	Added HCl, *N*	Recovery, %
0.000	52.7	0.833	100.3
0.002	64.7	1.270	100.1
0.021	94.5	1.740	100.2
0.105	99.4	2.082	99.2
0.210	95.1	3.000	99.8
0.434	97.2	4.000	99.7

The data show that the acidity of the solution should be above 0.1*N*, preferably from 0.5 to 4.0*N*. We have indicated above that the optimal concentration of hydrochloric acid in the acid extraction is from three to six normal. This range obviously includes that which gives optimal recoveries in the ether extraction.

In the last series the minimum time required for complete recovery of oxalic acid from acid solutions was determined. The 3*N* hydrochloric acid solutions of the extract from the previously described sample of spinach were used, the other conditions being those previously defined as optimum. The data in Table 4 indicate a minimum time of extraction with ether of 15 hours.

We have therefore adopted the following conditions for the acid and the other extractions: 1. Extraction of food sample with 3*N* hydrochloric acid for five hours. 2. Extraction of the resulting acid extract with ether for at least 15 hours. These conditions, employed as described in detail below, were also used to test the recovery of

TABLE 4

Effect of varying the time of ether extraction upon the percentage of oxalic acid in spinach

Extraction time	Oxalic acid	Extraction time	Oxalic acid
hours	%	*hours*	%
1	0.43	10	1.00
2	0.56	15	1.04
3	0.73	25	1.05
5	0.88		

additional amounts of standard oxalic acid. Aliquots of the latter were added to individual dry spinach samples and allowed to soak in. The samples were then redried and reground. While satisfactory recoveries were always obtained (98% and above) we cannot feel that the oxalic acid added in this way can be incorporated in the interior of the plant fibre as is that which has resulted from growth. Its quantitative recovery is therefore not, in our opinion, proof of similarly quantitative recovery of the naturally occurring acid. However, the data recorded above support our conclusion that optimum conditions for the determination of the naturally occurring oxalic acid have been demonstrated.

The remainder of the analysis, involving evaporation of the ether solution, conversion of the oxalic acid to calcium oxalate, and final titration with standard permanganate has been too thoroughly standardized for other purposes to require further study. This part of the determination is included in the detailed procedure described below, by which our figures were determined.

Detailed Procedure. The food was weighed fresh as purchased and then thoroughly dried, first in air at room temperature, then in an oven at 80°C. From the fresh and the dry weights, the moisture content was calculated. If the fat content, according to nutritional tables, was higher than 10% on the fresh basis, the food was defatted by extraction with carbon tetrachloride. The dried food was ground to pass through a 40-mesh screen and a representative portion of the dried and ground material was kept over phosphorus pentoxide in a desiccator until samples were taken for analysis. A 2- to 4-g. sample was placed in an extra-coarse sintered glass extraction thimble or crucible and extracted with 75 ml. of 3.0 to 4.0*N* hydrochloric acid in a Soxhlet extractor for 5 hours. The resulting acid extract was filtered through a "medium" porosity sintered glass funnel and the residue washed several times with distilled water. Filtrate and washings were made up to 100 ml. of volume. Twenty-ml. aliquots of this extract were then pipetted into the inner tube of a modified Clausen *(2)* extractor, using a straight 29- x 300-mm. pyrex tube with indentations to support the inner tube as described by Kyker, Webb, and Andrews *(8)*. The acid solution was then extracted with ether for 15 hours or more. The extract was transferred quantitatively into a 250-ml. Erlenmeyer flask by repeated rinsing of the outer extractor tube with distilled water and the ether evaporated. The aqueous solution remaining after removal of the ether was filtered into a 50-ml. centrifuge tube and the pH adjusted to approximately 7.0 with ammonium hydroxide and acetic acid using brom thymol blue as indicator. Saturated calcium chloride solution was added in an amount at least double the equivalent of the expected oxalate content. The solution was then maintained at approximately 60°C. overnight. After centrifuging, the supernatant solution was discarded and the precipitate dissolved in normal sulfuric acid. The solution was transferred quantitatively to a 250-ml. Erlenmeyer flask and titrated hot with standard potassium permanganate solution.

RESULTS AND DISCUSSION

Table 5 shows the results obtained on a variety of foods by the above described procedure. These figures are expressed in terms of the percentage of anhydrous oxalic acid in the fresh vegetable. The figures for oxalic acid in some miscellaneous foods at the end of the table are based on the food as purchased. It should be pointed out that these figures are an expression

TABLE 5

Oxalic acid (anhydrous) content of foods

Food	Source	Water	Oxalic acid found by	
			Present authors	Kohman
Vegetables		%	%	%
Asparagus stalks	Local	94.9	0.015	.....
Asparagus tips	Local	93.7	0.018	0.0052
Beans, green	Local	88.2	0.037	0.0310
Broccoli	Colorado	91.0	0.021	0.0054
Brussels sprouts	Florida	87.3	0.037	.....
Cabbage	Local	92.2	0.019	0.0077
Cauliflower	Florida	91.3	0.025	0.0
Celery	California	95.5	0.026	0.0340
Chives	Florida	69.9	0.185	.....
Collards	Local	81.5	0.074	0.0091
Corn, white, fresh	Local	80.2	0.041	0.0014
Cucumbers	Florida	96.9	0.004	0.0
Eggplant	Florida	96.7	0.018	0.0069
Endive	Florida	90.5	0.018	0.0273
Escarole	Florida	94.4	0.031	0.0116
Kale	Eastern N. Carolina	84.5	0.125	0.0130
Leeks	Florida	69.9	0.089	.....
Lettuce, head	California	95.5	0.011	0.0071
Okra	Local	84.5	0.146	0.0480
Onions, green	Local	89.9	0.076	0.023
Rhubarb	California	95.0	0.275	0.500
Spinach	Florida	86.7	1.050	0.892
Squash, summer	Local	94.8	0.022	0.0
Tomatoes	Georgia	96.0	0.013	0.0075
Turnip greens	Local	92.8	0.053	0.0146
Fruits				
Apples	Virginia	83.0	0.030	0.0
Strawberries	Local	94.2	0.047	0.019
Tubers				
Beets	Florida	90.6	0.127	0.138
Carrots	California	89.9	0.060	0.033
Parsnips	Florida	69.3	0.081	0.010
Potatoes, sweet	Local	69.6	0.141	0.056
Potatoes, white	Local	76.4	0.056	0.0057
Potatoes, white	Western N. Carolina	81.8	0.034	0.0057
Potatoes, white	Idaho	77.6	0.064	0.0057
Rutabagas	S. Carolina	87.6	0.019	.....
Radishes	Local	96.2	0.015	0.0
Turnips	Local	90.6	0.019	0.0018
Legumes				
Limas, fresh	Local	75.8	0.047	0.0043
Peanuts, raw	Virginia	8.0	0.225	.....
Peas	.....	75.0	0.006	0.0
Miscellaneous				
Chocolate, unsweetened	.....	.....	0.325	.....
Cocoa	.....	.....	0.908	.....
Pecans	.....	4.3	0.202	.....
Rice	.....	5.2	0.186	.....
Soybean crackers	.....	3.8	0.207	.....
Wheat germ	.....	.....	0.269	.....

of the total oxalate content of the food without regard to whether it occurs as the free acid or as a salt. Moreover, we have not, in this work, been concerned with the calcium content of the food.

Because of our interest in the geographical source of the food we have, where possible, ascertained its source. This information has not always been available nor have we been able to secure samples of the same food from a wide geographical range. However, we have recorded the source of the food, if known, in the table. Blank spaces indicate an unknown source. By "local" is meant the area within about 25 miles of Chapel Hill.

For convenience in comparison, we have included the oxalic acid figures published by Kohman *(6)* for those foods which we have studied. Although a number of our figures are in substantial agreement with those of Kohman, it is apparent that in many cases ours are very much higher than his, the only conspicuous exception being our figure for rhubarb. In this case we have obtained but little more than half of the 0.50% reported by Kohman. For this vegetable, Arbenz *(1)* reports 0.32%. For spinach, the outstanding source of food oxalates, Kohman reports a figure substantially in agreement with ours whereas the Arbenz figure (0.29%) is far below anything we have ever encountered in this vegetable.

It is interesting to note the higher figures we have obtained in spite of the fact that we do not credit the solid residue from the acid extraction as containing any further oxalic acid. In case of deficient acid extraction we should expect our figures to be too low. It might be argued that our failure to make the two or three reprecipitations of calcium oxalate described by Kohman could be responsible for high results. However, it is difficult to envisage the precipitation of any other calcium salt sufficiently insoluble to contaminate the calcium oxalate and capable of reducing permanganate. In addition, we have, in a number of cases, reprecipitated the calcium oxalate twice more without obtaining any significant decrease in the oxalic acid percentage which resulted from one precipitation. Had our high results been due to absorption of other reducing substances on the calcium oxalate, the fact should have been demonstrated by this procedure.

Of the foods of local origin, nearly all show an oxalate content much higher than that reported by Kohman. It should be recognized, however, that there may be many causes for this variation including type of soil, climate, and latitude. In particular, the studies of Wittwer, Albrecht, and Goff *(11)* have shown a direct relationship between the oxalate content of swiss chard and the nitrogen content of the soil.

SUMMARY

The method of estimation of oxalic acid in various foods has been studied and optimum conditions have been determined for both the acidity and the time of extraction in both the acid and the ether extractions.

The oxalic acid content, as estimated by our procedure, is reported for about 45 foods. With only four exceptions, our results are substantially higher than those of previous investigators. Seven of these among the fresh foods show contents of oxalic acid above 0.1%.

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INDICES OF POLLUTION IN OYSTERS[a]

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The occurrence of numerous outbreaks of typhoid fever due to the consumption of oysters taken from sewage-polluted waters has led to the promulgation of strict sanitary regulations concerning this popular seafood. The need for dependable methods for determining the sanitary quality of oysters is greater than with most seafoods because they are often eaten in the raw state, and the usual methods of cooking may not destroy intestinal pathogens *(1, 6)*. Also, the ability of the typhoid organisms to survive in oysters for many days (possibly several weeks) after they have been removed from polluted water and placed in clean water *(7, 8)* or in cold storage *(8, 18)* emphasizes the importance of adequate sanitary control of this product.

The use of the coliform test as a measure of the sanitary quality of oysters has had several critics who consider the *Aerobacter* section to have little significance because of their common occurrence in unpolluted areas. Perry *(14)*, Perry and Bayliss *(15)*, and Hunter *(4, 5)* have suggested the use of *Escherichia coli* as a more suitable indicator of dangerous pollution in oysters than the coliform group. In recent years there has been renewed interest in the possibility of employing the fecal streptococci as indicators of pollution *(2, 3, 9, 10, 12, 13, 16, 19, 20)*. Members of this group are constantly present in large numbers in sewage, apparently they do not multiply to any extent in nature, and they can be detected rapidly in food and water.

The purpose of this investigation was to determine the incidence of the enterococci, *Escherichia coli*, and the coliform group in oysters along the Louisiana coast, and to correlate these findings with the probability of pollution as determined by sanitary inspection.

METHODS

The oysters were taken from reefs along the Louisiana coast of the Gulf of Mexico, from Cameron Parish on the west to Plaquemines Parish on the east. The samples were collected by a sanitarian of the Louisiana Department of Health, who classified each oyster bed as to its sanitary condition. The task of the sanitarian in classifying oyster reefs as to probability of pollution was very difficult because of shifting camps of oil exploration crews. Those reefs not situated near the mouth of a bayou, and apparently not exposed to any type of pollution were designated as "clean." Reefs less favorably situated, but not exposed to obvious pollution were called "not polluted." Areas exposed to effluents from moderately polluted streams were said to be "moderately polluted." All the reefs except those designated as "moderately polluted" were used for commercial production of oysters.

The oyster samples were packed in ice and shipped by express to the laboratory, where they were carefully shucked and examined within 24 to 36 hours after the dredg-

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TABLE 1

Numbers of coliforms and enterococci in shell oysters from the Louisiana Gulf Coast

Sample No.	Quality[b]	MPN per gram		
		Coliforms	*E. coli*	Enterococci
6	NP	200	3.3	2.0
7	NP	35,000	350.0	0
8	NP	790	4.9	3.3
9	NP	24,000	79.0	7.9
10	NP	79,000	490.0	0.45
11	NP	70	2.0	0
12	NP	0	0	2.0
13	.....	130	33.0	1.8
14	NP	490	7.8	1.8
15	NP	1,700	2.0	4.5
16	NP	110	20.0	0
17	NP	33	2.0	0
18	NP	1,100	2.0	2.0
19	NP	11	0	0
20	NP	4.5	0	0
21	C	11	4.9	0
22	C	49	17.0	0
23	C	33	0	0
24	C	22	0.2	0
25	MP	33	3.3	0
26	MP	23	2.0	0.2
27	NP	33,000	14.9	0
28	NP	79	13.0	0
29	NP	3,000	13.0	49.0
30	NP	240	79.0	0
31	MP	22	4.9	1.1
32	MP	330	330.0	4.6
33	NP	49	2.0	0.2
34	NP	49	3.3	0.2
35	C	70	1.3	0.2
36	C	4	2.3	0
37	Q	920,000	540.0	790.0
38	C	54,000	130.0	13.0
41	C	2.0	0.2	4.5
42	C	33	0	0
43	C	33	2.0	0
44	C	13	0.4	0
45	C	22	0	0
46	MP	4.5	0.2	0
47	MP	7.8	1.3	0

[b] Sanitary condition as reported by a sanitarian of the State Department of Health. NP, not polluted; MP, moderately polluted; C, clean; Q, questionable.

ing operation. The oysters were thoroughly cleaned with soap and brush, rinsed in running tap water and allowed to drain, and then they were opened in the usual way with a sterile oyster knife. A sample for bacteriological examination consisted of at least 10 oyster bodies lifted out of the shell without draining, and weighing at least 100 grams. The shell liquor was not added to the sample, but was separately examined in some of the tests.

The samples were macerated in a Waring blendor with an equal weight of sterile water, and appropriate quantities were introduced into the various media for the determination of the numbers of bacteria. The standard plate counts were determined with tryptone glucose extract agar (Difco) using an incubation temperature of 37°C. for 48 hours. The enterococci were determined with the method of Winter and Sandholzer *(19, 20)* using 5 replicate tubes of each dilution in the presumptive test. Confirmation was with the slant-broth medium of Winter and Sandholzer *(19, 20)*. Organisms that produced pinpoint colonies on the slant, sediment in the broth, and were gram positive cocci were considered to be enterococci. Coliforms were detected with lauryl sulfate tryptose lactose broth, using 5 replicate tubes of each dilution. Confirmation was in brilliant green lactose bile (2%), and the completed test was as prescribed in "Standard Methods for the Examination of Water and Sewage" *(17)*. The Hajna and Perry *(3)* modification of the Eijkman test was employed for the enumeration of *E. coli*. Five replicate tubes were employed in the presumptive test; confirmed and completed tests were performed as indicated above for the coliform group. Identification of *E. coli* was by means of the "Imvic" pattern. Most probable numbers (MPN) were obtained as suggested in "Standard Methods for the Examination of Water and Sewage" *(17)*.

TABLE 2

Incidence of coliforms, *E. coli*, and enterococci in shell oysters

Range of MPN per g.	Percentage of samples containing the indicated number of		
	Coliforms	*E. coli*	Enterococci
0-5	12.5	67.5	90.0
5.1-25	20.0	15.0	5.0
25.1-100	27.5	5.0	2.5
101-500	15.0	10.0	0
501-5,000	10.0	2.5	2.5
5,001-100,000	12.5	0	0
Over 100,000	2.5	0	0

RESULTS

The results obtained with 40 samples of shell oysters collected from the Louisiana coast are presented in Table 1. The numbers (MPN) of coliforms varied from 0 to 920,000 per gram of oysters, and the numbers of *E. coli* and enterococci varied from 0 to 540 and 0 to 790, respectively. *E. coli* was not found in 6 samples (15%) and enterococci were not detected in 21 samples (52.5%). One sample lacking *E. coli* contained enterococci, and 16 samples containing *E. coli* did not contain enterococci.

Of the 40 samples examined, only 4 contained more than 5 enterococci per gram. *E. coli* was encountered more frequently and in larger numbers; 32.5% of the samples contained more than 5 *E. coli* per gram (Table 2). The total coliforms were much more abundant; only 12.5% of the samples contained less than 5 per gram and 40% contained over 100 per gram.

In a number of instances parallel examinations were made of the shell liquors and the oyster bodies. The numbers of the various groups of bacteria found in these samples are indicated in Table 3. The polluted sample

TABLE 3

Bacteria in the liquors and bodies of shell oysters

Sample No.	Plate count/g.	MPN per gram		
		Coliforms	*E. coli*	Enterococci
35 Liquor	11,900	7.8	0.2	0
Body	12,550	70.0	1.3	0.2
36 Liquor	2,020	0	.....	0
Body	3,700	4.0	.....	0
37 Liquor	.....	160,000	220	160
Body	.....	920,000	540	790
38 Liquor	239,000	11,000	79	35
Body	230,000	54,000	130	13
41 Liquor	3,300	0	0	0
Body	900	2	0.2	4.5
42 Liquor	2,800	33	0	0
Body	5,400	33	0	0
43 Liquor	230,000	170	1.3	0.2
Body	630,000	33	1.3	0
44 Liquor	31,000	330	1.3	0.2
Body	22,000	13	1.3	0
45 Liquor	55,000	23	0	0
Body	7,500	22	0	0
46 Liquor	11,000	0.2	0.2	0
Body	13,000	4.5	0.2	0
47 Liquor	39,000	0.2	0.2	0
Body	7,000	7.8	1.3	0

TABLE 4

Relationship of numbers of *E. coli* and enterococci to coliforms in shell oysters

Range of Coliforms per g.	Number of samples	Average MPN per gram		
		Coliforms	*E. coli*	Enterococci
0-25	13	11	1.4	0.6
26-50	8	39	3.7	0.05
51-100	3	73	5.4	0.06
101-1,000	7	327	68.2	1.9
1,001-10,000	3	1,933	.....	.....
Over 10,000	6	190,800	267.3	136.0

(No. 37) contained a significantly larger number of all three indicator organisms in the oyster bodies than in the shell liquors. In no instance was *E. coli* more numerous in the shell liquor than in the oyster bodies, and in only 2 samples were coliforms significantly more numerous in the shell liquors. On the basis of these results it seems that the use of the oyster shell liquor for the bacteriological examination is not essential.

TABLE 5

Relationship of enterococci and total coliforms to *E. coli* in shell oysters

E. coli per g.	Number of samples	Average MPN per gram		
		E. coli	Enterococci	Coliforms
0-2	19	1.0	0.7	170
2.1-5	7	3.8	1.0	158
5.1-25	6	14.3	8.5	6,121
26-250	4	80.2	5.7	19,592
Over 250	4	427.0	198.7	258,582

In the samples studied there were wide variations in the relationship between the numbers of coliforms, *E. coli*, and enterococci. There was, however, a general relationship between the various groups, as indicated in Tables 4 and 5. Those samples containing relatively few bacteria of one group also contained relatively few organisms of the other groups.

TABLE 6

Indicator organisms in shell oysters from reefs of different sanitary classes

Sanitary classification	Number of samples	Average MPN per gram		
		Coliforms	*E. coli*	Enterococci
Clean	12	4,524	13.3	1.50
Not polluted	20	8,961	86.5	3.60
Moderately polluted	6	70.6	56.9	0.98
Questionable (polluted)	1	920,000	540.0	790.0

In Table 6 the samples are grouped according to the probability of pollution as estimated by the sanitarian. The bacteriological results confirmed his evaluation of the "clean" oysters; the distinction between the "not polluted" and the "moderately polluted" samples does not appear to be justified by the bacteriological findings. The sample classed as "questionable" by the sanitarian was obviously polluted, and was the only sample containing large numbers of all three indicator organisms.

DISCUSSION

In this study a general correlation was found between the numbers of the coliform group and the numbers of *E. coli* and the enterococci. Since *E. coli* is generally considered the best bacterial indicator of fecal pollution, it may be assumed that any organisms which inhabit the intestinal tract and whose numbers in foods are closely correlated with *E. coli*, may be employed as indicators of pollution. Both the coliform group and the

enterococci possess these requisites. In shell oysters the coliform group was found in much larger numbers than *E. coli*, but, if quantitative values were considered, the distinction between the clean and the polluted oysters was almost as clearly indicated by the one as by the other. If the coliform group should be employed as the pollution indicator the maximum tolerance would of course have to be much higher than if *E. coli* were used as the indicator organism.

The enterococci were present in much smaller numbers in shell oysters than the coliforms and *E. coli*, and were not found in over half the samples. However, their numbers were directly correlated with *E. coli*, hence they too may be employed as indicators of pollution in shell oysters. These organisms possess certain advantages over the coliform group as pollution indicators, since they are found in large numbers in sewage and feces, and apparently do not multiply in soil and water as the coliforms sometimes do. The enterococci seem to possess the same advantages over the coliform group as has been claimed for *E. coli* by Hunter *(4)*, Perry *(14)*, and Perry and Bayliss *(15)*. If an indicator organism is sought whose mere presence may be considered as strongly suggestive of pollution, the enterococci are more satisfactory than *E. coli* and *E. coli* is better than the coliform group.

The correlation we have found between the enterococci and *E. coli*, and the ease and rapidity with which the enterococci can be determined should lead to further studies on these organisms in shell oysters. Our results suggest that the enterococci test may be used to advantage with the *E. coli* determination in evaluating the sanitary quality of oysters. If either organism is absent and the other is present in small numbers, one may be confident the oysters are of excellent quality; if both organisms are present in considerable numbers one may be sure the oysters are not of good sanitary quality.

The results obtained when the pooled shell liquors and oyster bodies were separately examined suggest that the usual practice of examining both liquor and bodies is not essential. The indicator organisms were usually more numerous in the bodies than in the liquors, but the differences were in most instances small.

CONCLUSIONS

Shell oysters taken from the Louisiana coast of the Gulf of Mexico were found to contain coliform group organisms almost invariably; 67.5% of the samples contained 100 or more coliforms per gram. *E. coli* was found in small numbers in most samples; 67.5% of the samples contained less than 5 *E. coli* per gram. Ninety percent of the samples contained less than 5 enterococci per gram; none were found in 52.5% of the samples. The numbers of each group of indicator organisms were usually larger in the oyster bodies than in the shell liquor.

Oysters with large numbers of coliforms usually contained relatively large numbers of *E. coli* and enterococci, and the converse was also true. The enterococci appeared to be useful as indicators of pollution, and it is suggested they be used along with *E. coli* in further studies.

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CALCIUM CONTENT OF COMMERCIAL BREAD

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The action of the Food and Nutrition Board of the National Research Council to increase the recommended allowances for calcium, focused attention on the food sources of this essential element. Because of the provision for calcium as an optional ingredient in the standard of identity for enriched bread, and because of the widespread consumption of this food, consideration was given to making calcium enrichment at a minimum level of 300 mg. per pound mandatory.

In 1939, Prouty and Cathcart *(4)* reported the results of a survey of 39 loaves of white bread obtained from 33 bakeries located in 27 states. These investigators found the average calcium content to be 0.080%, or 363 mg. per pound of bread on a 38% moisture basis. Earlier reports on the calcium content of bread, as summarized by Prouty and Cathcart, revealed lower values, indicating that there was an upward trend in the use, by bakers, of ingredients which contribute significant amounts of calcium.

For this reason, it was deemed desirable to determine the calcium content of commercial bread and to determine, insofar as possible, the source of the calcium in bread, especially the amount contributed by nonfat milk solids.

A survey using a questionnaire approach, conducted by Cook *(3)*, indicated that the proportion of milk to flour used by commercial bakers in the manufacture of bread in 1948 was equal to that used in 1939, and was somewhat in excess of 4 parts of milk for every 100 parts of flour. Rogers *(5)*, however, has stated that the average use of nonfat milk solids in commercial bread is only 1.5 parts for every 100 parts of flour. He reached his value from the following calculations *(6)*:

"In Bulletin 107 was given an estimate of the approximate level of milk solids in bread which we believed to be accurate at the time, and, as we mentioned previously, it was based on census figures and communications from milk producers directly supplying the baking industry. Additional published evidence that the 1.5% level is a fair approximation may be realized by considering that according to the 1949 volume of Agricultural Statistics, the per capita consumption of flour in this country was 135 pounds, and with a population of 150 million, 20,250,000,000 pounds of flour were consumed. If we allow 1.5 pounds of milk solids for each 100 pounds of flour, as a tentative level, some 303,750,000 pounds of milk solids would be required. Of course, a portion of the flour consumed has no added milk solids whatsoever, but there is also another portion of the total flour which is enriched to a higher level than the 1.5 figure. Obviously these two portions tend to offset each other. With these figures, the total milk solids used by the baking industry is 1.55% of the flour consumed. To account for the 303,750,000 pounds of milk solids which would be needed at the 1.5 level, we find that 223,929,000 pounds of dried milk solids was used by the baking industry (1947 Census of Manufacturers, Bakery Products, page 8), approximately 55,000,000 pounds of milk solids were supplied in the form of sweetened condensed milk products (BDIM-Inf-107, page 9), 30,000,000 pounds of milk solids were supplied as condensed

milk (BDIM-Inf-107, page 9), and 2,400,000 pounds of milk solids as fluid milk (BDIM-Inf-107, page 9). If a similar calculation is made for the amount of milk solids that would be needed for a 3% level, an amount is found which is far beyond the published figures for milk solids production."

EXPERIMENTAL

Samples and Methods Used. For the study to be reported, samples of wrapped, sliced white or enriched bread from 135 bakeries located in 34 cities, and 23 samples of whole wheat bread from 23 cities, and 12 samples of wheat bread from 12 cities (made from various combinations of whole wheat and white flours), were purchased on the open market during the winter months of 1949-50 and shipped Air Mail to the Institute Laboratories for analysis. Services of Western Union were used in procuring these samples. With the exception of six samples, all of the white bread received was enriched.

The moisture content of the samples as received was determined by the A.O.A.C. *(1)* method. The calcium content was determined using the ash of duplicate 10 g. samples of the ground, air-dried bread and the permanganate titration method of Shohl and Pedley *(7)*. The lactose content of the bread was determined by the method of Choi, Tatter, and O'Malley *(2)*, and was used as an index of milk content. The percent of nonfat milk solids in the bread was calculated on the basis of 50% lactose in nonfat milk solids. The calcium contribution of the milk was calculated using the factor of 1.24%, as the calcium content of nonfat milk solids. By employing the fact that 65% of the bread weight represents the amount of flour used in average commercial formulas, the percent of nonfat milk solids based on flour weight was calculated.

RESULTS AND DISCUSSION

The results calculated on a uniform basis of 38% moisture content of the bread are given in Tables 1, 2, and 3. The average calcium content of enriched bread and white bread was 404 mg. per pound, as compared with the average calcium content of 365 mg. per pound for whole wheat bread, and 348 for wheat bread, despite the higher calcium content of whole wheat flour over white flour.

Only fifteen samples of white bread or enriched bread fell below 300 mg. calcium per pound. The average calcium content of these 15 samples was 236 mg. per pound. It would appear that revision of the standards of identity for enriched bread, to make mandatory a minimum calcium level of 300 mg. per pound, would increase the intake of this element only slightly.

The average milk content of the samples of white bread and enriched bread analyzed, calculated as nonfat milk solids on 38% moisture basis, was 2.4% as compared to 1.6% nonfat milk solids in the wheat and whole wheat bread. The percent of milk in bread is usually expressed in bakers' formulas as parts of milk for each 100 parts of flour. When calculated in this manner, it is found that the average baker's formula for white and enriched bread contains 3.8% nonfat milk solids, while the average formula for wheat and whole wheat bread contains 2.4% nonfat milk solids.

The results of these analyses agree with the results obtained by Cook using a questionnaire technique, but differ from the figure published by Rogers. However, the method of calculation, used by Rogers, is subject to criticism and his results, therefore, cannot be a reflection of the baker's practice.

In making his calculations, Rogers used the national per capita consumption of flour and the amount of milk solids used by the baking indus-

TABLE 1

The calcium and milk content of commercial white bread

Sample number	Where purchased, city and state	Bread composition on 38% moisture basis			Nonfat milk solids in formula, parts/100 parts of flour
		Milk nonfat milk solids, %	Mg. of calcium contributed from milk/lb. bread	Total calcium content, mg./lb.	
	New England and Middle Atlantic States				
1	Boston, Massachusetts	3.0	169	335	4.6
2	Boston, Massachusetts	1.2	77	368	1.7
3	Boston, Massachusetts	2.4	132	327	3.7
4	Boston, Massachusetts	2.1	116	281	3.2
5	Providence, Rhode Island	2.2	121	410	3.4
6	Providence, Rhode Island	1.7	98	306	2.6
7	Providence, Rhode Island	2.8	159	485	4.3
8	Providence, Rhode Island	3.0	169	356	4.6
9	New Haven, Connecticut	1.8	99	411	2.7
10	New Haven, Connecticut	2.7	153	503	4.2
11	New Haven, Connecticut	2.0	111	346	3.1
12	New Haven, Connecticut	2.8	156	553	4.3
13	New York, New York	2.1	120	436	3.2
14	New York, New York	3.6	99	548	5.5
15	New York, New York	1.9	107	440	2.9
16	New York, New York	3.3	188	431	5.1
17	Brooklyn, New York	1.9	109	330	2.9
18[a]	Brooklyn, New York	2.8	157	217	4.3
19	Brooklyn, New York	2.3	192	515	3.5
20	Brooklyn, New York	3.4	131	429	5.1
21	Philadelphia, Pennsylvania	3.3	188	434	5.1
22	Philadelphia, Pennsylvania	2.7	151	433	4.2
23	Philadelphia, Pennsylvania	2.7	152	334	4.2
24	Philadelphia, Pennsylvania	2.2	121	299	3.4
25	Pittsburgh, Pennsylvania	3.6	205	498	5.5
26	Pittsburgh, Pennsylvania	2.3	132	420	3.5
27	Pittsburgh, Pennsylvania	2.8	156	431	4.3
28	Pittsburgh, Pennsylvania	2.2	126	213	3.4
	South Atlantic States				
29	Washington, D. C.	2.2	122	368	3.4
30	Washington, D. C.	1.9	108	355	2.9
31	Washington, D. C.	3.0	173	608	4.6
32	Washington, D. C.	2.9	159	444	4.5
33	Richmond, Virginia	4.0	225	324	6.2
34	Richmond, Virginia	2.6	149	327	4.0
35	Richmond, Virginia	1.7	98	412	2.6
36	Richmond, Virginia	3.8	211	545	5.8
37	Charleston, South Carolina	2.1	117	452	3.2
38	Charleston, South Carolina	3.4	192	425	5.1
39	Charleston, South Carolina	1.9	107	378	2.9
40	Charleston, South Carolina	2.6	144	195	4.0
41	Atlanta, Georgia	2.7	151	494	4.2
42	Atlanta, Georgia	4.1	232	425	6.3
43	Atlanta, Georgia	2.3	127	517	3.5
44	Atlanta, Georgia	3.7	208	429	5.7
45	Miami, Florida	2.1	119	324	3.2
46	Miami, Florida	2.3	129	492	3.5
47	Miami, Florida	2.0	113	446	3.1
48	Miami, Florida	3.5	199	444	5.4

TABLE 1 *(Continued)*

Sample number	Where purchased, city and state	Bread composition on 38% moisture basis			Nonfat milk solids in formula, parts/100 parts of flour
		Milk nonfat milk solids, %	Mg. of calcium contributed from milk/lb. bread	Total calcium content, mg./lb.	
	North Central States				
49	Cleveland, Ohio	2.1	121	253	3.2
50	Cleveland, Ohio	2.9	161	362	4.5
51	Cleveland, Ohio	3.1	173	343	4.7
52	Cleveland, Ohio	3.0	169	313	4.6
53	Cincinnati, Ohio	2.6	149	631	4.0
54	Cincinnati, Ohio	3.0	167	532	4.6
55	Cincinnati, Ohio	2.7	151	435	4.2
56	Cincinnati, Ohio	1.3	73	237	2.0
57	Muskegon, Michigan	3.6	204	590	5.5
58	Muskegon, Michigan	3.7	206	432	5.7
59	Muskegon, Michigan	1.9	106	520	2.9
60	Muskegon, Michigan	2.1	117	325	3.2
61	Detroit, Michigan	2.9	162	356	4.5
62	Detroit, Michigan	1.8	101	427	2.7
63	Detroit, Michigan	3.6	204	356	5.5
64	Detroit, Michigan	2.2	123	494	3.4
65	Chicago, Illinois	2.9	163	410	4.5
66	Chicago, Illinois	3.6	203	495	5.5
67	Chicago, Illinois	2.9	163	328	4.5
68[a]	Chicago, Illinois	0	0	199	0
69	Minneapolis, Minnesota	3.7	207	540	5.7
70	Minneapolis, Minnesota	1.9	105	385	2.9
71	Minneapolis, Minnesota	3.6	201	534	5.5
72	Minneapolis, Minnesota	3.3	186	324	5.1
73	Madison, Wisconsin	2.4	136	398	3.7
74	Madison, Wisconsin	0.4	23	313	0.6
75	Madison, Wisconsin	2.6	144	404	4.0
76	Madison, Wisconsin	2.0	114	347	3.1
77	Kansas City, Missouri	2.4	114	345	3.7
78	Kansas City, Missouri	3.4	192	484	5.1
79	Kansas City, Missouri	1.6	91	419	2.5
80	Kansas City, Missouri	2.5	141	440	3.8
	South Central States				
81	Louisville, Kentucky	3.1	177	511	4.7
82	Louisville, Kentucky	1.6	92	337	2.5
83	Louisville, Kentucky	2.1	118	463	3.2
84	Louisville, Kentucky	1.4	79	228	2.2
85	Chattanooga, Tennessee	0.5	34	352	0.8
86	Chattanooga, Tennessee	2.3	128	359	3.5
87	Chattanooga, Tennessee	3.3	186	368	5.1
88	Memphis, Tennessee	2.0	114	351	3.1
89	Memphis, Tennessee	2.1	120	301	3.2
90	Memphis, Tennessee	3.5	195	516	5.4
91	Memphis, Tennessee	1.9	107	418	2.9
92	New Orleans, Louisiana	3.0	170	375	4.6
93	New Orleans, Louisiana	2.4	133	334	3.7
94	New Orleans, Louisiana	3.0	169	590	4.6
95	New Orleans, Louisiana	2.5	139	354	3.8
96	Oklahoma City, Oklahoma	1.4	77	329	2.2
97	Oklahoma City, Oklahoma	3.5	196	367	5.4
98	Oklahoma City, Oklahoma	2.4	136	463	3.7

TABLE 1 *(Concluded)*

Sample number	Where purchased, city and state	Bread composition on 38% moisture basis			Nonfat milk solids in formula, parts/100 parts of flour
		Milk nonfat milk solids, %	Mg. of calcium contributed from milk/lb. bread	Total calcium content, mg./lb.	
99	Oklahoma City, Oklahoma	2.9	164	562	4.5
100	Dallas, Texas	2.1	172	336	3.2
101	Dallas, Texas	2.0	110	366	3.1
102	Dallas, Texas	2.4	135	540	3.7
103	Dallas, Texas	2.2	125	327	3.4
104	El Paso, Texas	2.5	142	401	3.8
105	El Paso, Texas	1.5	85	428	2.3
106	El Paso, Texas	1.0	57	336	1.5
107	El Paso, Texas	3.4	193	590	5.2
108	Houston, Texas	1.8	100	438	2.7
109	Houston, Texas	1.9	104	363	2.9
110	Houston, Texas	1.2	65	406	1.9
111	Houston, Texas	2.6	149	419	4.0
	Mountain and Pacific States				
112	Denver, Colorado	3.5	196	420	5.4
113	Denver, Colorado	2.1	121	374	3.2
114	Denver, Colorado	3.1	175	359	4.7
115	Denver, Colorado	2.8	159	361	4.3
116	Salt Lake City, Utah	3.8	216	541	5.8
117	Salt Lake City, Utah	3.3	186	391	5.1
118	Salt Lake City, Utah	2.7	152	572	4.2
119	Salt Lake City, Utah	2.5	139	401	3.8
120	Portland, Oregon	2.3	129	399	3.5
121	Portland, Oregon	0.7	38	190	1.1
122[a]	Portland, Oregon	3.9	220	441	6.0
123	Portland, Oregon	1.8	104	368	2.7
124	San Francisco, California	3.0	169	294	4.6
125	San Francisco, California	0.5	27	230	0.7
126	San Francisco, California	3.8	211	638	5.8
127[a]	San Francisco, California	0.8	45	213	1.2
128	Los Angeles, California	1.1	59	500	1.7
129	Los Angeles, California	3.7	206	442	5.7
130	Los Angeles, California	0.6	36	279	0.9
131	Los Angeles, California	3.0	170	572	4.6
132	Sacramento, California	1.9	109	348	2.9
133[a]	Sacramento, California	1.0	55	242	1.5
134	Sacramento, California	1.3	72	369	2.0
135	Sacramento, California	3.1	172	584	4.7
	Average	2.4	138	404	3.8

[a] Wrapper did not carry label statement of enrichment.

TABLE 2

The calcium and milk content of commercial whole wheat bread

Sample number	Where purchased, city and state	Bread composition on 38% moisture basis			Nonfat milk solids in formula, parts/100 parts of flour
		Milk nonfat milk solids, %	Mg. of calcium contributed from milk/lb. bread	Total calcium content, mg./lb.	
	New England and Middle Atlantic States				
1	New Haven, Connecticut	3.1	173	470	4.8
2	New York, New York	3.5	197	453	5.4
3	Brooklyn, New York	2.7	155	283	4.2
4	Pittsburgh, Pennsylvania	1.5	81	344	2.3
	South Atlantic States				
5	Washington, D. C.	0	0	384	0
6	Richmond, Virginia	2.6	147	317	4.0
7	Atlanta, Georgia	0	0	396	0
8	Miami, Florida	0	0	321	0
	North Central States				
9	Cleveland, Ohio	0	0	401	0
10	Muskegon, Michigan	2.2	122	338	3.4
11	Chicago, Illinois	1.5	83	377	2.3
12	Minneapolis, Minnesota	3.7	209	464	5.7
13	Madison, Wisconsin	0	0	286	0
	South Central States				
14	Louisville, Kentucky	1.1	64	461	1.7
15	New Orleans, Louisiana	0	0	252	0
16	El Paso, Texas	1.7	94	382	2.6
17	Houston, Texas	2.7	149	336	4.2
	Mountain and Pacific States				
18	Denver, Colorado	2.1	121	444	3.2
19	Salt Lake City, Utah	2.6	145	340	4.0
20	Portland, Oregon	1.7	93	295	2.6
21	San Francisco, California	0.7	40	410	1.1
22	Los Angeles, California	0.7	42	295	1.1
23	Sacramento, California	2.0	110	336	3.1
	Average	1.57	88	365	2.4

try. The per capita consumption of flour figure used by Rogers includes, besides the flour used by the commercial baker, all of the household flour, flour sold to other industries manufacturing food for man and animals, and flour sold to industries employing flour for non-food purposes.

The Census of Manufacturers reveals that the Baking Industry used less than 50% of the flour consumed during the period for which Rogers made his calculations, or 9,702,300,000 pounds. Calculations based on this quantity of flour and the amount of milk solids used by the baker during the same period indicate the average milk content of baked foods, including bread but not limited to bread, would be 3.2%.

Approximately one-third of the calcium found in white or enriched white bread was derived from the milk used in the formulae; while only one-fourth of the calcium found in wheat and whole wheat bread could be attributed to this source. Sources of calcium in commercial bread and their calcium contributions, when used in customary amounts, are given

TABLE 3

The calcium and milk content of commercial wheat bread

Sample number	Where purchased, city and state	Bread composition on 38% moisture basis			Nonfat milk solids in formula, parts/100 parts of flour
		Milk nonfat milk solids, %	Mg. of calcium contributed from milk/lb. bread	Total calcium content, mg./lb.	
	New England and Middle Atlantic States				
1	Boston, Massachusetts	0.9	50	300	1.4
2	Providence, Rhode Island	2.2	123	373	3.4
3	Philadelphia, Pennsylvania	1.5	81	344	2.3
	South Atlantic States				
4	Charleston, South Carolina	2.3	127	501	3.5
	North Central States				
5	Cincinnati, Ohio	1.0	57	301	1.5
6	Detroit, Michigan	2.1	118	428	3.2
7	Chicago, Illinois	1.6	90	316	2.5
8	Kansas City, Missouri	0	0	352	0
	South Central States				
9	Chattanooga, Tennessee	2.7	152	210	4.2
10	Memphis, Tennessee	0.3	2	354	0.5
11	Oklahoma City, Oklahoma	1.0	58	267	1.5
12	Dallas, Texas	3.1	172	435	4.8
	Average	1.56	86	348.4	2.4

in Table 4. It is to be noted that some of the adjuncts, such as some of the mold and rope inhibitors (calcium propionate and calcium acid phosphate), and yeast foods, used by the baker mainly as production aids, actually enhance the food value of the finished loaf.

TABLE 4

Sources of calcium in bread

Ingredient	Amounts in a typical formula, lb.	Calcium contributed to each lb. of bread, mg.
Flour (white)	100	56
or		
Whole wheat flour	100	112
Water	60	15(0.3-30)
Nonfat milk solids	4	148
Yeast foods	0.25-0.50	112
Calcium propionate	0.32	203
Monocalcium phosphate	0.75	275

CONCLUSIONS

Analyses of bread samples indicate that bread is a significant source of calcium in the diet. White and enriched bread contribute somewhat more calcium (404 mg. per pound) than does whole wheat bread (365 mg. per pound) or wheat bread (348 mg. per pound). Significant amounts of this calcium are contributed by milk solids, which were found to average

3.8 parts per 100 parts of flour in white and enriched bread, and 2.4 parts per 100 parts of flour in wheat and whole wheat bread.

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THE EFFECT OF EXPERIMENTALLY INOCULATING CANNED BREAD WITH SPORES OF *CLOSTRIDIUM BOTULINUM* AND *BACILLUS MESENTERICUS*[a,b]

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The hazard of botulism as well as the problem of spoilage must be considered when the heat processing of foods in closed containers is inadequate to destroy heat resistant bacterial spores. During the development of canned bread for the Armed Forces, these problems were of foremost importance due to the conditions of handling and the time and temperature of baking the bread. Experiments carried out in the laboratories of the American Can Company showed that *Clostridium botulinum*, type A, would grow and produce toxin in canned bread which did not contain mold inhibitors, or in cans containing inhibitors which leaked and took up water during cooling. Therefore, the present investigation was carried out to determine the factors limiting the germination and growth of spores of *C. botulinum* in canned bread and to establish the conditions necessary for the safe production of the bread. Since *Bacillus mesentericus*, causing ropy bread, is a common spoilage organism in bread, this organism was included in the study to determine its effect on the growth and toxin production of *C. botulinum*. The quantity and distribution of moisture in the bread loaves was investigated since it is known that the amount of moisture present in dehydrated meat may effect the growth and toxin production of *C. botulinum*, Segalove and Dack *(7)*.

MATERIALS

Two different formulae were used for preparing the bread doughs, one having a higher pH and moisture content (the old formula) than the other (the new formula); both formulae are given in Table 1.

The following strains of *C. botulinum* were used in this study:

Strain	Type	Obtained from
457	A	Dr. L. S. McClung, Indiana University
62A	A	Dr. K. F. Meyer, Hooper Foundation, University of California
109	A	Dr. K. F. Meyer, Hooper Foundation, University of California
169	B	Dr. K. F. Meyer, Hooper Foundation, University of California
12033	B	National Canners Association
126.7	B	Department of Bacteriology, The University of Chicago

The *B. mesentericus* used was strain 4 obtained from the Fleischmann Laboratories, New York City.

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[a] The experiments reported in this investigation were carried out in cooperation with the staff of the Quartermaster Food and Container Institute for the Armed Forces and with representatives of the American Can Company and the Continental Can Company.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 327 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

TABLE 1

Old and new bread formulae

Ingredients	Old formula	Based on weight of flour, %	New formula	Based on weight of flour, %
Flour[c]	12 lb., 8 oz.	100.0	12 lb., 8 oz.	100.0
Salt	3½ oz.	1.75	3½ oz.	1.75
Sugar	8 oz.	4.0	8 oz.	4.0
Shortening[d]	8 oz.	4.0	8 oz.	4.0
Milk powder[e]	4 oz.	2.0	4 oz.	2.0
Monocalcium phosphate	½ oz.	0.25	½ oz.	0.25
Calcium propionate	¼ oz.	0.125	.....	.....
Yeast, compressed	4 oz.	2.0	4 oz.	2.0
Water	7 lb., 8 oz.	60.0	6 lb., 12 oz.	54.0
Sodium diacetate (Dykon)[f]	.....	.....	1⅛ oz.	0.56
Edible lactic acid (80%)[g]	.....	.....	7.1 ml.	0.125

[c] Low protein, short patent, hard winter wheat.
[d] Hydrogenated shortening, 100-hr. stability.
[e] Nonfat milk solids.
[f] Manufactured by Stein Hall and Company, Inc. Composition as follows: 70% sodium diacetate; 15% starch; 15% calcium sulphate (34% acetic acid).
[g] Manufactured by E. I. du Pont de Nemours.

PRELIMINARY EXPERIMENTS

Effect of Size of Inoculum. Before the actual experimental work with bread doughs was started, the effect of the size of the inoculum was determined in experiments designed to test sterilized bread as a medium for the growth and toxin production of *C. botulinum*. Tubes of sterilized old and new formula breads and beef heart medium (control) were inoculated with 0, 3,000, 300,000, or 30,000,000 spores of *C. botulinum*, strain 62A, mixed with 0, 3,000, 300,000, or 30,000,000 spores of *B. mesentericus*. The breads were mixed with saline in a weight ratio of one part bread to four parts saline and autoclaved before inoculation. The *C. botulinum* spores were heated at 80°C. for 15 minutes immediately before inoculating to destroy vegetative cells and botulinum toxin, Coleman and Meyer *(1)*. The tubes were incubated anaerobically *(4)* in a desiccator jar containing 10% CO_2 at 37°C. for 10 days before testing by intraperitoneal mouse injection for toxin production. *C. botulinum* toxin was present in all the tubes inoculated with *C. botulinum* spores; but no deaths occurred in mice injected with the supernatant from the tubes inoculated only with *B. mesentericus* spores.

Effect of Moisture. Under normal conditions some cans may be imperfectly sealed and during cooling by a water spray or immersion in a tank of water may take in water through faulty seams. This necessitated an experiment to test the effect of increased moisture on the growth and toxin production of *C. botulinum* in bread. Six cans of bread were experimentally inoculated in the center of the loaf with a syringe containing spore suspensions of either strain 62A *C. botulinum* or a mixture of *C. botulinum* and *B. mesentericus*. The cans were then immersed in a water bath and punctured under water with a steel pin. The pin produced a hole about 1.0 mm. in diameter. The cans were immersed for an hour and then removed, wiped dry and sealed with a solder. All cans were held for 10 days; they were incubated at 37°C. unless bulging was observed and then they were placed at room temperature. After opening, enough sterile saline was added so that the total liquid volume per can was approximately 600 ml. No determination was made of the water uptake due to immersion, nevertheless the product was soggy and unedible.

Mice were injected intraperitoneally with 1.0-ml. amounts of undiluted and decimal dilutions in saline of the supernatant fluid. Only one of the six cans contained *C. botulinum* toxin. This was an old formula bread inoculated with only *C. botulinum* spores. Two guinea pigs each were given 2 ml. of this bread suspension by stomach tube. One pig also received 0.5 ml. type A antitoxin intraperitoneally. The test pig died within 2 days. One-ml. amounts of diluted bread were also inoculated into beef heart medium to test for viable spores of *C. botulinum*. The beef heart cultures were heated at 80°C. for 10 minutes after which they were incubated at 37°C. for 10 days. Following incu-

bation, the cultures were centrifugalized at high speed for 30 minutes, and the clear supernatant fluids removed for toxicity tests in mice. Mice received 1.0 ml. of undiluted supernatant fluid intraperitoneally, and control mice were similarly injected with the same material plus 0.1 ml. of homologous antitoxin. Viable *C. botulinum* spores were recovered from one old and one new formula loaf inoculated with *C. botulinum* and one old formula bread inoculated with *C. botulinum* and *B. mesentericus*.

The effectiveness of the mixing procedure in the detection of small amounts of toxin in a localized area was tested. Two cans of bread were inoculated approximately in the middle of the loaves with 1.0 ml. and 0.1 ml., respectively, of supernatant fluid from a seven- to eight-day culture of *C. botulinum* in casein digest broth. After mixing the breads with saline and allowing them to stand for about 15 minutes, 1.0 ml. and 0.1 ml. from each can were injected into mice. One-tenth ml. of the original culture was also injected into a mouse. Death occurred in all mice tested.

EXPERIMENTS WITH BREAD MADE FROM DOUGH INOCULATED WITH SPORES OF C. BOTULINUM AND B. MESENTERICUS

Experimental Procedure. After the completion of the above preliminary experiments, one old and two new formula batches of bread were prepared. The sponge water used in making the doughs for each batch was inoculated with a saline spore suspension of *C. botulinum* so that each loaf of bread (10½ oz. baked weight) would contain one million spores. The old formula bread and one batch of new formula bread were inoculated with strain 62A; the other new formula batch was inoculated with the six previously mentioned strains of *C. botulinum* spores. In addition, some of the doughs of each batch were inoculated with a saline spore suspension of *B. mesentericus* so that each loaf of bread would contain 25,000 spores of *B. mesentericus* in addition to the one million *C. botulinum* spores. One uninoculated batch of new formula bread was also prepared as a control.

The natural source of *B. mesentericus* may be contaminated flour. Hoffman, Schweitzer, and Dalby *(3)* found in a study covering a period of several years that the maximum *B. mesentericus* spore count for flour was 150 spores per gram. The inoculum of *B. mesentericus* spores used approximated 140 spores per gram of flour which is above the acceptable standard for flour and was considered ample to cause "ropy" bread in the absence of inhibitors, since O'Leary and Kralovec *(6)* reported that "ropy" bread was produced with an inoculum of six *B. mesentericus* spores per gram of flour.

The breads from each batch inoculated with *C. botulinum* spores were divided into three groups and stored with uninoculated new formula loaves at 22, 32, and 38°C. (72, 90, and 100°F.), respectively. The breads inoculated with both *C. botulinum* and *B. mesentericus* spores were stored at 32°C.(90°F.). All cans incubated at 32°C.(90°F.) were subjected to weekly cycling at 4.5°C.(40°F.) for 16 hours, following which they were returned to the 32°C.(90°F.) incubator. This cycling was done to stimulate practical storage conditions favoring condensation of moisture in portions of the can.

Three uninoculated loaves, five loaves inoculated with the six *C. botulinum* strains, and five loaves inoculated with the six *C. botulinum* strains and *B. mesentericus* were tested the day after baking. With a few exceptions, 10 cans from each of the storage sets were examined for *C. botulinum* toxin and ropiness after one and two weeks, and after 1, 3, 6, 12, 18, and 24 months. One uninoculated can stored at each temperature was removed at each time interval and used as a pH control.

All the cans were tested for vacuum immediately before storage and upon removal from storage by means of a "flip tester" supplied by the Continental Can Company. In the analysis of the bread, the end of the can having the headspace was opened after the top of the can had been cleaned and sterilized by flaming. The bread was then mixed in the can with a large spoon with approximately 600 ml. of sterile 0.85% saline. Since there were approximately 300 g. of bread per can, the final dilution was 1:3. The can was covered with a sterile glass plate and allowed to stand until some of the solids settled. Samples of the supernatant fluid were then drawn off for pH determinations and mouse inoculation tests. All bread cans and samples were stored in the refrigerator until needed.

One-ml. samples of the supernatant fluid from each loaf were injected intraperitoneally into mice. If death occurred within four days, the sample was re-examined with a test and a control mouse. In the case of bread samples inoculated with the single *C. botulinum* strain, 0.1 ml. of type A antitoxin was used for the control mouse. For the samples of bread inoculated with the six *C. botulinum* strains, three controls were used; one mouse was given type A antitoxin, the second type B, and the third a polyvalent ABC antitoxin.

B. mesentericus counts were made on some of the loaves by plating out the bread saline mixture in nutrient agar. These plates were incubated at 37°C. for five days and then counted.

The approximate number of viable *C. botulinum* spores was determined in random samples taken from the various lots of bread. Two samples taken from each of the sets of ten loaves analyzed at each period were opened aseptically and mixed under a hood. Dilutions were made and 1.0 ml. of these inoculated into 9 ml. of beef heart medium. In addition, in most cases, 10 ml. of the 1:3 bread dilution were inoculated into flasks containing 100 ml. of beef heart. Immediately after inoculation, these tubes and flasks were heated at 80°C. for 10 to 15 minutes to destroy any toxin which may have been present. They were then incubated at 37°C. for 10 days. *C. botulinum* toxin production was taken as evidence for the presence of viable spores in the bread. If death in the mice occurred due to toxic metabolic products other than *C. botulinum* toxin, the supernatant fluid was diluted 1:10 and again injected.

RESULTS

Physical Appearance of Non-Toxic Loaves. The vacuum in the cans, the appearance, consistency, and odor of all the bread loaves, and the pH of the saline bread mixtures were recorded. Only 20 of 1003 loaves tested showed a considerable loss in vacuum and could properly be considered leakers. This loss of vacuum occurred some time after 24 hours from the time of sealing the cans, since a normal vacuum was found in all the cans when "flip tests" were made after the cans were cooled. Some of these loaves and a few additional ones had an off odor, however, with the exceptions to be noted later, none of these loaves contained botulinum toxin.

One week after baking, the cut surfaces of both the old and new formulae bread appeared almost white. After 18 months of storage, the cut surfaces of the old formula bread had a golden cast, while the new formula bread was much lighter in color. Only an occasional can of bread prepared by either formula turned a dark brown. At the one-month and the succeeding test intervals, black spots appeared on the bread along the can seam. As time progressed, these spots occasionally turned red, green, or white and were sometimes damp. Counts for *B. mesentericus* organisms were made on several of these spots at each time interval. The spot areas were removed, emulsified in saline, and pour plates were made. *B. mesentericus* organisms were recovered from these spots after 3, 6, and 12 months, but not after 18 or 24 months. In general, there was no evidence of multiplication of *B. mesentericus* in these spots.

Presence of Botulinum Toxin. Only three cans of bread contained botulinum toxin as shown by mouse inoculation. These were old formula bread loaves inoculated with spores of both *B. mesentericus* and *C. botulinum*, strain 62A, and stored at 32°C.(90°F.) for six months with weekly cycling at 4.5°C.(40°F.). A slight fall in vacuum was noted in the 10 cans of this group tested at the six-month interval, and these loaves had a yeast odor (at three months the odor was sour and cheesy). It was noted after three months that the loaves of this group had soft centers. As the incubation

time progressed, these centers became damp and larger in size. After 18 months, the lower crusts were soft and damp. The soft, mushy area appeared to be cone-shaped. No further changes were observed at the 24-month interval.

B. mesentericus counts were made on the centers of some of these loaves at each storage interval indicated. The following were the number of organisms (average for specimens tested) per gram of bread: six months, 3600; 12 months, 2100; 18 months, 1125; 24 months, 0. The original inoculum of *B. mesentericus* spores was 85 spores per gram. Many of these spores, however, were probably destroyed during the baking procedure.

Viable Spore Count. The results of the viable spore counts are given in Table 2. It is interesting to note that type B *C. botulinum* spores were not recovered after the first week of incubation.

pH. The pH values of the bread samples were measured on a Beckman potentiometer. The original pH of the old formula bread was about 5.4 and during incubation fluctuated between pH 4.62 and 5.6. For the new formula bread, the initial pH 4.9 was obtained and during incubation the extremes of variation were pH 4.33 and 5.45. In general, the variation was slight and usually there was not more than 0.3 variation of a pH during incubation and storage.

Moisture. Some of the canned bread prepared by the old formula and inoculated with the spores of *C. botulinum* and *B. mesentericus* developed ropy centers after six months of storage. The question then arose as to whether this moisture was produced by the hydrolysis of starch by *B. mesentericus* or whether it was transferred from one portion of the canned bread to another. In an attempt to answer this question, experiments were carried out at the American Can Company Research Laboratories.[k] An experimental lot of bread was made using an old formula bread dough, without inhibitors. The dough was inoculated by adding to the yeast water approximately 10 grams of finely powdered bread crumbs having 100,000 spores of *B. mesentericus* per gram (Fleischmann Laboratories rope preparation No. 4/6A/682). This amounted to the addition of 90 spores per gram of flour or 6,850 spores per can of bread (4½ oz. can). The ingredients used in the preparation of an old formula bread were: flour, 400 ounces; sugar, 8 ounces; milk S.N.F., 8 ounces; shortening, 8 ounces; salt, 7 ounces; yeast, 8 ounces; and water, 232 ounces.

The day after baking, six cans were analyzed for moisture content, and the number of *B. mesentericus* spores per gram of bread determined on an additional six cans. The remaining cans were held at 32°C.(98°F.) until examination after 35 and 98 days. An examination was also made on three of these cans of bread and an additional three from a lot stored at 22°C.(72°F.) after 231 days.

The moisture determination was made as follows on six cans of bread for each examination except after 231 days of storage when three were examined. After the gauge vacuums were taken, the cans were weighed to the nearest 0.01 gram on an analytical balance, opened, and placed in a 70°C. vacuum drying oven over night. The cans and the bread were again weighed to the nearest 0.01 g. after which the bread was removed and the empty cans were weighed. The bread was ground, and a small sample

[k] Experiments conducted by Messrs. Hays, Alexander, and Wade.

TABLE 2

Presence of viable spores of *Clostridium botulinum*[a] in 1:3 dilutions of canned bread

(Two cans of each sample tested at each interval)

Storage time	Spore inoculum	Storage temp. °F.	Inoculated with spores of one type A strain				Inoculated with spores of 3 type A and 3 type B strains	
			No. cans having viable spores in 1-ml. samples		No. cans having viable spores in 10-ml. samples		No. cans having viable spores in 1-ml. samples	No. cans having viable spores in 10-ml. samples
			OF[b]	NF	OF	NF	NF	NF
0 (Control)	*C. botulinum*	.....	....	....	....	....	0	2[c]
	C. bot. and *B. mes.*	.....	....	....	....	....	1	2
1 week	*C. botulinum*	72	1	0	....	....	0	2
		90	2	0	....	....	0	2[c]
		100	2	0	....	....	0	2
	C. bot. and *B. mes.*	90	2	1	....	....	0	2
2 weeks	*C. botulinum*	72	2	0	....	....	0	2
		90	1	1	....	....	0	0
		100	2	0	....	....	0	1
	C. bot. and *B. mes.*	90	2	1	....	....	0	2
1 month	*C. botulinum*	72	1	0	....	....	0	1
		90	2	0	....	....	0	1
		100	1	0	....	....	0	2
	C. bot. and *B. mes.*	90	2	0	....	....	0	2
3 months	*C. botulinum*	72	0	0	2	0	0	1
		90	2	0	2	1	0	2
		100	0	0	2	0	0	1
	C. bot. and *B. mes.*	90	2	0	2	2	0	2
6 months	*C. botulinum*	72	2	2	2	2	0	2
		90	2	2	2	2	0	2
		100	2	2	2	2	0	2
	C. bot. and *B. mes.*	90	2	1	2	2	0	2
12 months	*C. botulinum*	72	0	0	2	2	0	2
		90	0	0	2	0	0	1
		100	0	2	2	1	0	0
	C. bot. and *B. mes.*	90	1	0	2	2	0	2
18 months	*C. botulinum*	72	2	2	2	2	0	1
		90	2	1	2	1	0	2
		100	2	2	2	0	0	1
	C. bot. and *B. mes.*	90	2	2	2	2	0	0
24 months	*C. botulinum*	72	2	1	1	2	2	2
		90	2	1	2	1	0	2
		100	2	1	2	1	0	0
	C. bot. and *B. mes.*	90	2	0	2	2	0	1

[a] Only type A *C. botulinum* spores recovered; no type B spores recovered, except where indicated by [c].

[b] OF, old formula; NF, new formula.

[c] One of two cans contained both type A and type B viable *C. botulinum* spores.

weighed into an aluminum moisture dish and dried at 100°C. under vacuum to constant weight. The original moisture content was computed from the weight losses found in the second and third weighings. Six cans of bread were examined for ropy areas at the one-month and succeeding examinations. In those loaves of bread having ropy areas, ropy bread spots were weighed into tared moisture dishes and dried to constant weight under vacuum at 100°C. During the 98- and 231-day examinations, normal areas of the loaves adjacent to the ropy areas were weighed into tared moisture dishes and dried to constant weight under vacuum at 100°C.

The number of spores of *B. mesentericus* was determined on six cans during each examination. After an unopened can was weighed, it was opened and the loaf cut aseptically into small pieces with a sterile knife. Three hundred ml. of sterile water was added to the bread which was then disintegrated in a sterile mixer cup. One-ml. and decimal dilutions of the resulting bread slurries were pipetted into petri dishes, and pour plates made with dextrose tryptone agar. The plates were incubated at 37°C. (98°F.) for 48 hours. After the 24-hour examination, additional ropy areas of the six loaves of bread used for the moisture determinations were collected and added aseptically into weighed sterile water blanks. The weight of the samples was determined. The samples were shaken vigorously and one-ml. and decimal dilutions plated as above. The weight of the bread was determined by subtracting the weight of the empty can from that of the unopened can.

There was a considerable spread between the *B. mesentericus* counts per gram of bread of the six cans examined the day after baking. The counts ranged from 35 to 319 per gram of bread or from somewhat more than half of the calculated number (60 per gram of bread) introduced by the inoculum to about five times that number. While the average *B. mesentericus* count of the whole loaves of bread increased during three months of storage from 206 to 3,100,000, the average *B. mesentericus* count of ropy areas of bread decreased rapidly after the first month from 167,000,000 to 28,000,000 after three months.

There is an apparent shift of moisture from the normal areas to the ropy areas as is evidenced by an average moisture of 36.44% for all loaves examined as compared to 44% for ropy areas and 32.39% for the normal bread adjacent to the ropy areas.

B. mesentericus Metabolism. Since botulinum toxin was found only in bread inoculated with both *C. botulinum* and *B. mesentericus* spores, the question arose as to whether the metabolic products of *B. mesentericus* influence the growth of *C. botulinum*. A possibility was that *B. mesentericus* hydrolyzes starch to dextrose which may be used by *C. botulinum*.

Experiments[1] were carried out to determine whether the growth of *B. mesentericus* in bread resulted in an accumulation of dextrose. The *B. mesentericus* strain used was found to hydrolyze starch. Old formula bread was used. A one to three dilution (by weight) of bread was made in distilled water and homogenized in a Waring blendor. Samples of 210 g. of this material (70 g. of bread) were placed in each of eight flasks and autoclaved for 20 minutes at 15 pounds of steam pressure. Then 125 g. more water was added to each flask and the mixtures shaken to break up clumps as much as possible. Four flasks were then inoculated with 0.5 ml. of a 24-hour broth culture of *B. mesentericus*. Four uninoculated flasks were used as controls. The flasks were rotated on a wheel while being incubated

[1] Experiments conducted by Drs. M. S. Bergdoll and H. Sugiyama in the Food Research Institute.

at 37°C. After 19 hours of incubation, two inoculated and two control flasks were removed.

Upon examination, the contents of the inoculated flasks were found to be "mushy" in consistency as compared to the controls, and to contain only vegetative cells. Smears made of the contents of the two remaining inoculated flasks after five days of incubation showed numerous spores, therefore on the sixth day, the remaining flasks were removed from the incubator.

Five hundred ml. of ethanol was added to extract the sugars from the bread mixture. The mixtures were centrifuged and the residues re-extracted five times with 65% ethanol. The solutions were concentrated to remove the ethanol. Ten ml. of a saturated solution of neutral lead acetate were added to the concentrated fluid and the volume made up to one liter and filtered through a layer of Hyflo-Super-Cel. Anhydrous Na_2CO_3 was added to the filtrates to precipitate the excess lead, and the solutions were filtered through a layer of Hyflo-Super-Cel. Five-ml. aliquots were diluted to 100 ml., and then 10 ml. of this dilution was taken for the determination of total reducing sugars by the method of Phillips *(7)*. Five-ml. aliquots were used (made to 20 ml.) for fermentations according to the method of Phillips and Smith *(8)*, and 5 ml. of the fermented solutions were used for reducing sugar determinations.

TABLE 3

Reducing sugars before and after growth of *Bacillus mesentericus*

	Average[m] total reducing sugar (as glucose), g.		Average[m] non-fermenting reducing sugar (as glucose), g.		Fermenting reducing sugar (as glucose), g.		Net increase, in grams[n]	
	19 hrs.	6 days	19 hrs.	6 days	19 hrs.	6 days	19 hrs.	6 days
Test	4.24	3.16	2.56	3.03	1.68	0.13	0.12	.....
Control	2.22	2.20	0.417	0.510	1.80	1.69	.....	−1.56

[m] Duplicate determinations were made on two test and two control samples at each time interval.
[n] Difference in grams between control and test fermenting reducing sugar values.

In the bread preparation inoculated with *B. mesentericus* spores there was a slight decrease in reducing sugars after 19 hours of incubation. After approximately six days, there was an almost complete loss (Table 3). This would indicate that reducing sugars were either not produced or that they were utilized as they were produced. From these results it is doubtful if the growth of *C. botulinum* can be attributed to glucose resulting from the hydrolysis of starch by *B. mesentericus*.

DISCUSSION

Of the 1003 loaves of canned bread prepared, 976 were inoculated with spores and only three cans of old formula bread contained botulinum toxin. Therefore, canned bread does not appear to be a good medium for the germination, growth, and toxin production of *C. botulinum*. The two principal differences between the old and the new formula bread were moisture content and pH. Of these two variables the most important was moisture. Toxin was produced when *C. botulinum* type A spores were inoculated into old and new formula breads whose moisture content was increased by the addition of saline. It has been previously observed in the case of dehydrated meat that growth and toxin production of *C. botulinum* occurred when the moisture content was 40% but not 30% *(9)*. The bread had

approximately 34% moisture when prepared by the new formula as compared with 37% moisture in the old formula.

The pH in general was higher after six months and fluctuated somewhat after that. Since the low pH values permit feeble growth of *C. botulinum*, acidity of the bread does not eliminate the botulism hazard.

The evidence would indicate that *C. botulinum* spores had not germinated since the counts were relatively constant throughout the 24-month observation period. With the new formula, fewer spores of *C. botulinum* appeared to survive the storage conditions. In the three cans in which botulinum toxin was demonstrated, there was no increase in the number of spores of *C. botulinum*. The possibility cannot be ignored, however, that an increase in the number of spores might have occurred, but that the spores failed to grow in the recovery culture medium, or the spores may have died during the storage period. Other explanations for the production of toxin without an increase in the spore count may be that the spores germinated and subsequently multiplied but did not form new spores, the vegetative cells either dying during the storage period or being destroyed during the exposure to air in making dilutions for the counts or being killed during the heating in making the spore counts. It has previously been demonstrated by Dack and Williston *(2)* and Leifson *(5)* that actively growing vegetative cells of *C. botulinum* do not grow when diluted and placed in favorable medium.

Multiplication of *B. mesentericus* occurred only in the old formula bread and became macroscopically apparent only after six months of storage as shown by the ropiness of the centers of the loaves. In the entire study, *C. botulinum* toxin was found in only three of these ropy loaves and these at the six-month interval. There was a shift in the moisture from the non-ropy areas so that the ropy areas had from 9 to 14% higher moisture than non-ropy, although the total moisture of the whole loaf remained constant. It is appreciated, however, that the vapor tension is the same throughout the can and that the difference would be represented in bound and free water. Whether this moisture relationship is responsible for the growth and toxin production of *C. botulinum* in the three cans of toxic bread is a matter of conjecture; the relationship between *C. botulinum* toxin production and growth of *B. mesentericus* is similarly uncertain. The activity of *B. mesentericus* apparently was self-limited, since at 24 months no viable *B. mesentericus* was demonstrated. It is also doubtful whether the toxin production of *C. botulinum* in the ropy bread can be attributed to glucose resulting from the hydrolysis of starch by *B. mesentericus*.

SUMMARY

Canned bread is a non-sterile product. *C. botulinum* remained viable over a two-year storage period in canned bread experimentally inoculated with spores of types A and B. In some experiments where the experimental inoculum was a mixture of spores of *C. botulinum* and *B. mesentericus*, the spores of *C. botulinum* were viable over the same storage period. The cans were stored at 22 and 38°C.(72 and 100°F.); others were stored at 32°C.(90°F.) with a weekly cycling at 4.5°C.(40°F.) for periods of 16 hours. Although the spores remained viable, there was no evidence of

growth or toxin production except after six months of storage. Three of ten cans of old formula bread experimentally inoculated with *C. botulinum* spores, strain 62A, in addition to *B. mesentericus* spores and stored at 32°C.(90°F.) and cycled were toxic when tested in mice. In the case of these toxic cans, the experimental inoculum with spores of *B. mesentericus* was greater than naturally found in raw ingredients.

It is concluded that canned bread is a poor medium for growth and toxin production of *C. botulinum*. Moisture appeared to be the limiting factor. In no case was toxin demonstrated in the new formula bread loaves tested. Under the conditions of these experiments the preponderance of evidence indicates that the hazard of botulism is probably negligible in the new formula bread. However, positive automatic procedures should be used to eliminate from distribution cans which have failed to be hermetically sealed and may later permit the moisture content of the bread to increase. In addition, close positive control should be maintained over original bread moisture content, pH, and contamination with spores of *B. mesentericus*.

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MAPLE SIRUP. III. PRELIMINARY STUDY OF THE NONVOLATILE ACID FRACTION[a]

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Relatively little scientific work has been done to improve the quality and uniformity of maple sirup or to develop improved processing methods. Much of the literature *(2, 5, 12)* relates to control measures, such as prevention of adulteration and detection of contamination. Since the value of maple sirup lies in its distinctive flavor, a better knowledge of the flavoring substances is necessary for improvement of processing practices. Maple sirup consists mainly of sucrose and water; the flavoring material is present among the trace constituents. Some investigations on maple flavor have been reported *(2, 3, 5)*, but the results were not complete. Findlay and Snell *(3)* reported that the flavor is developed during heat evaporation of sap to sirup. Our preliminary work demonstrated that sap as it leaves the maple tree has no maple flavor. An essentially flavorless sirup was produced by vacuum evaporation at room temperature, confirming the observation of Findlay and Snell. We further confirmed it by production of a completely flavorless sirup by freeze drying. When heated at atmospheric pressure, this sirup acquired typical maple flavor, proving that during the production of sirup the flavor is developed by heating the maple sap at atmospheric pressure.

These facts were the basis of our hypothesis that maple flavor is the result of a browning (Maillard) reaction. Production of a synthetic sirup having a maple-like flavor by Barnes and Kaufman *(1)* lends support to this assumption. Production of high-flavored maple sirups by heat treatment of commercial maple sirups *(11)* may be further evidence of the role of the browning reaction.

To investigate the possibilities of the browning reaction between amino acids, sugars, organic acids, and aldehydes, it was necessary to study in detail each of these groups as it occurs in maple sap. The progress report given here on the nonvolatile acid fraction is the first of a series of papers dealing with the composition of maple sap and maple sirup and is the third of a general series of papers on maple sirup issued from this laboratory *(11)*.

The literature on the nonvolatile organic acids of maple sirup indicates that there is considerable uncertainty as to both the total number and the identity of these acids *(7, 9)*. Most of the acids reported were identified by the time-honored method of (a) extraction with ether after addition of acid equivalent to the total alkalinity of the ash, (b) distilla-

[a] Report of a study made under the Research and Marketing Act of 1946.

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tion of the volatile acids and precipitation of the nonvolatile acids as the lead salts, and (c) acid recovery by esterification and fractionation of the esters *(7)*. These procedures, even for large volumes of starting material (35 to 40 liters), may not be of sufficient sensitivity and accuracy to detect the acids existing in trace amounts. Methods employed in this work, in which only a fraction of the above amount of sirup was used were (a) ion-exchange techniques to separate the organic acids in a relatively pure form, and (b) paper chromatography for identification of the acids.

EXPERIMENTAL

Ion Exchange Methods. Sixty ml. of cation resin, Dowex 50,[c] in the spherical form was employed in the (H^+) ion cycle in a 17 x 0.75-inch ion-exchange tube equipped for downflow exhaustion and regeneration and for backwashing at 50% bed expansion. A flow rate of 0.26 ml. per minute per ml. of resin was used throughout the experiments. For regeneration, four bed volumes of 2*N* HCl were used, and the columns were washed with distilled water until the eluate was free of chloride ion.

Sixty ml. of anion resin, Duolite A-4, was used for the acid exchange. The bed volume, column dimensions, and rate of flow were the same as for the cation column. For regeneration, four bed volumes of 1*N* NaOH were used, and the columns were washed with distilled water until the eluate was acid to phenolphthalein.

Paper Chromatographic Methods. The method of Lugg and Overell *(6)* was followed with some variations. Strips of Whatman No. 1 paper (6 x 22.5 inches) were used as the solvent support. Downflow operation, with 3 hours of equilibration of the papers with the aqueous phase, was employed in all cases. After the papers were dried in a hood for 2 hours at room temperature, the spot positions of most of the acids were identified by spraying with bromophenol blue (0.04%) adjusted to slight alkalinity. Naphthoresorcinol (0.1%) in 1*N* alcoholic H_3PO_4 was used to indicate the presence of keto and uronic acids. Ammonium vanadate (2%) in water indicated the presence or absence of tartaric acid by the characteristic orange spot. Ammoniacal silver nitrate sprays produced characteristic colors with some acids after heating at 105°C. for 10 minutes, and additional information was obtained by viewing the papers, processed with this reagent, under ultraviolet light. Ninhydrin (0.1%) in n-pentanol was used for amino acids. Separate chromatograms were made for each detecting (spray) material.

Procedure. Two hundred g. of a commercial maple sirup was diluted with water to 15% solids content and run into the cation column at the stated flow rate, and the effluent was passed through the anion column immediately. The two columns were washed in series with distilled water until the effluent was free of acids and of sugars. The cation column was eluated with 2*N* HCl and washed with water. After concentration in vacuo, this eluate contained the inorganic cations and the neutral and basic amino acids. The organic acids retained by the anion column were eluted with 1*N* NaOH, and the column was washed with water until the eluate was acid to phenolphthalein. The eluate and washings containing the sodium salts of the organic acids were passed through a regenerated cation (H^+) column to form the free acids. These were washed from the column with distilled water, washing being continued until the eluate was free of acid.

The acid fraction was concentrated by boiling until test papergrams indicated the acid concentration that would give the best resolution. This also removed the volatile acids; any insoluble material formed was removed by filtration. Then 0.01 ml. of the acid concentrate was placed on the paper by means of a Breed and Brew pipette, and after the solvent had evaporated, the paper was hung from the solvent tray in a chromatographic tank containing only the aqueous phase of the solvent system obtained from the mutual saturation of equal volumes of n-pentanol and 5*M* formic acid. After the papers were equilibrated with the vapor of the water phase, the chromatograms were developed for 18 hours (the organic phase of the solvent system was used), removed from the tank, dried, and sprayed with the appropriate detecting solution. R_F values

[c] Mention of commercial products does not imply that they are indorsed or recommended by the Department of Agriculture over others of similar nature not mentioned.

were calculated for each spot. Final identification was made by comparison of each spot with that obtained from a known compound.

Table 1 shows the number of nonvolatile acids in the anion fraction separated by the papergrams, the R_F value of each spot, the R_F values of the reference compounds, the method of indicating spot positions, and whether reported previously in the literature. These results were obtained using a commercial No. 2 grade maple sirup.

TABLE 1

Nonvolatile organic acids in maple sirup

Spot No.	R_F	Acid	R_F value of reference compound	Indicator[a]	Presence reported in literature
1	0.03	Unidentified	.....	1, 2	.....
2	0.07	Unidentified	.....	3	.....
3	0.11	Unidentified	.....	1, 2	.....
4	0.16	Unidentified	.....	1, 4	.....
5	0.26	Citric	0.24	1, 5	Nelson(7)
6	0.36	Malic	0.35	1, 5	Nelson(7)
7	0.44	Glycolic or 1:2 dihydroxybutyric[b]	0.45	1, 5	No
8	0.47	Unidentified	.....	1	.....
9	0.50	Unidentified	.....	1	.....
10	0.64	Succinic	0.63	1, 5	Nelson(7)
11	0.68	Unidentified	.....	1	.....
12	0.82	Fumaric	0.82	1, 5	Nelson(7)

[a] Indicators: 1, bromophenol blue; 2, naphthoresorcinol in alc. H_3PO_4; 3, ninhydrin; 4, ammonium vanadate; 5, ammoniacal silver nitrate.

[b] Subject to final proof of identity by isolation and analysis.

DISCUSSION

Four of the twelve acids shown to be present in commercial maple sirup have been identified by their R_F values as citric, malic, succinic, and fumaric acids. The acid in spot 1 is a uronic acid, as indicated by the slow rate of movement and by the color produced with naphthoresorcinol. The acid in spot 7 could be glycolic acid or 1:2 dihydroxybutyric acid. Isolation of this compound is necessary for final confirmation. The acid in spot 2 is probably an amino acid, as indicated by the ninhydrin reaction, and is probably dicarboxylic, since it is not held by the cation ion exchanger under these experimental conditions. It has a low R_F value. This compound, however, may be a glycoside of the type suggested by Gottschalk and Partridge *(4)* formed during browning. Nelson reported the presence of tartaric acid, which should chromatograph with an R_F of 0.16. A spot with such an R_F was found but not identified as tartaric acid, since a negative result was obtained with the ammonium vanadate spray. Tricarballylic acid has been reported in the calcareous precipitate formed during maple sirup production *(8)* but not in maple sirup *(7)*, and no trace of it was found in our work. Qualitative studies with Fancy, No. 1, and No. 3 sirups indicate the same acids to be presnt, although probably in different proportions. Work is being continued on the isolation and identification of the remaining acids by chromatography, X-ray diffraction, ultimate analyses, and spectrophotometric examination.

SUMMARY

In a study of the browning reaction in the formation of maple flavor, the composition of the nonvolatile organic acid fraction of maple sirup was investigated by means of ion-exchange and paper chromatography. The presence of citric, malic, succinic, and fumaric acids has been confirmed. Glycolic acid or 1:2 dihydroxybutyric acid is possibly present. Further work on the isolation and identification of this compound is in progress. The absence of tartaric acid and tricarballylic acid has been suggested. The techniques employed are sensitive to the acids present in trace amounts and there has been no experimental evidence to indicate alteration of the naturally occurring acids.

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EFFECTS OF FOUR COOKING PRESSURES ON COMMERCIALLY FROZEN PEAS: COOKING TIME, PALATABILITY, ASCORBIC ACID, FOLIC ACID, THIAMINE, AND RIBOFLAVIN

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The purpose of this study was to determine the effect of increased temperature on the total cooking time and palatability, and on the retention of ascorbic acid, folic acid, thiamine, and riboflavin in commercially frozen peas. The cooking pressures used were 0 (boiling), 5, 10, and 15 pounds (gauge).

At the time this study was started, it was thought that the basic defect in pernicious anemia was a physiological failure to liberate free folic acid from the conjugates. Since some assay procedures such as that of Luckey, Briggs, Moors, Elvehjem, and Hart *(12)* employ heat treatment to aid in the liberation of conjugated forms of folic acid, one objective was to determine if the amount of free folic acid increased with the higher cooking temperatures. Also, folic acid has been reported to be heat labile; therefore, another objective was to determine the effect of increased cooking temperatures on the total folic acid retentions. Pure solutions of thiamine and riboflavin are unstable in the presence of heat.

The ascorbic acid content of frozen peas has been reported in the literature from as low as 11.6 mg. per 100 g. by Harris, Mapson, and Wang *(8)* and by Briant, MacKenzie, and Fenton *(2)* to as high as 28.9 mg. per 100 g. by Maloney *(15)*.

No reports were found in the literature on the free folic acid content of peas. However, a few reports were found on the total folic acid content. Lipke *(10)* reported the folic acid content of frozen peas as 10.4 γ per 100 g. using *Streptococcus faecalis*. Olson, Burris, and Elvehjem *(18)* reported the folic acid content of one sample of fresh peas as 59 γ per 100 g. using *Streptococcus faecalis*, and as 50 γ per 100 g. using *Lactobacillus casei*.

The reported thiamine content of frozen peas has ranged from 0.137 to 0.432 mg. per 100 g. *(4)*.

The riboflavin content of frozen peas has been reported from as low as 0.08 mg. by Briant, MacKenzie, and Fenton *(2)* to as high as 0.22 mg. per 100 g. by Fellers, Esselen, and Fitzgerald *(6)*.

Total ascorbic acid retention during the cooking of frozen peas has ranged from 82 by Johnston, Schauer, Rapaport, and Deuel *(9)* to 99% by Maloney *(15)*. No report of the retention of folic acid during the cooking of frozen peas was found in the literature. The retention of thiamine in peas and cooking water has ranged from 84% as reported by Richardson and Mayfield *(19)* to no loss by Barnes, Tressler, and Fenton *(1)*, Maloney *(15)*, and McIntosh and Jones *(14)*.

The retention of riboflavin in the drained peas and in the cooking water has been found as 83 to 88% of the fresh raw content by Van Duyne, Wolfe, and Owen *(20)* and from 97 to 103% by Maloney *(15)*.

EXPERIMENTAL

History of the Frozen Peas. The frozen peas used in this study were all from one lot and were "run of the garden." They had been packed by Birds Eye-Snider Division, General Foods Corp., New York. The case was purchased from a retailer in Ithaca, New York, and was stored in a home-type freezer at −17° to −19°C. until the peas were used in the cooking studies.

A second lot of frozen peas from the same packer, to be used in a supplementary study, was purchased from the same retailer and stored in the same manner as above.

Cooking. Prior to each cooking, the packages of peas were removed from the freezer and held at room temperature only until an internal temperature of 0°C. was reached.

The peas were cooked in a four-quart aluminum pressure saucepan. In the studies employing atmospheric pressure, a glass cover was substituted for the pressure cover. In the pressure cooking a thermometer was placed in a rubber stopper, which was inserted in the lid of the pan after the automatic safety valve was removed. The gas pressure was controlled by a manometer in the manner of Fenton, Tressler, Camp, and King *(7)*.

In each study, frozen peas (with their adhering frost) in 300 g. lots were cooked. This amount represented four servings. In the instances where no pressure and 5 lb. pressure (gauge) were used, the peas were cooked in the adhering frost. When cooking with 10 lb. of pressure, 5 ml. of tap water was used, and with the 15 lb. pressure cooking, 10 ml. of tap water was used. These amounts of water were added so that there would be a minimum amount of water left at the end of each cooking and so that results therefore could be attributed to pressure alone and not to a combination of pressure and ratio of cooking water to vegetable. Cooking times are given in Table 1.

TABLE 1

Time required to cook frozen peas at four different pressures

Cooking pressure, lb. (gauge)	Cooking times required			
	To reach 100°C.[a]	From 100°C. to pressure	At pressure[b]	Total[c]
	min.	*min.*	*min.*	*min.*
(Atmospheric)	7 7/12	.....	1 5/12	9
5	7 7/12	2	1/4	9 5/6
10	7 7/12	2 1/12	.....	9 2/3
15	7 7/12	2 1/4	.....	9 5/6

[a] Time for the water to come to the boil.
[b] At the end of times given, cold tap water was allowed to run over the cover. As soon as the pressure was brought to zero the cover was loosened and the peas allowed to stand 10 minutes before they were sampled.
[c] Time that the peas were over the heat.

At the end of the cooking the temperature was rapidly reduced to 100°C. by holding the pressure saucepan under running cold water; the weight was removed and the cover opened. The peas were allowed to stand in the pan with the cover placed on loosely for 10 minutes before they were sampled. This was done to simulate conditions in homes where the peas are transferred to a serving dish, taken to the table and there served on the individual dinner plates. This 10-minute holding period allowed any cooking water that was present to be absorbed by the peas, or to evaporate, and thus eliminated the analysis of cooking water. The peas were weighed just prior to analysis.

A supplementary study was made to determine the ascorbic acid loss during the 10-minute holding. The frozen peas were cooked as described above at atmospheric pressure. In two cookings the drained peas and the cooking water were sampled imme-

diately; in the other two cookings the peas were sampled after a 10-minute holding period as described above.

Palatability. Peas cooked by each method were scored for appearance, texture, and flavor by six judges. Maximum score for each of the quality factors was five. A summation score of 15 was equivalent to "excellent"; 12, "good"; 9, "fair"; 6, "poor"; and 3, "very poor."

Analytical Methods. Reduced ascorbic acid was determined by the indophenol-xylene extraction method of Nelson and Somers *(17)*. Folic acid was assayed with *Streptococcus faecalis*, with a modification of the method of Luckey, Briggs, and Elvehjem *(11)*, as adapted by Carlson *(3)*. A chick pancreas preparation was used to liberate the conjugated folic acid. Thiamine was determined by the modified thiochrome method of Conner and Straub *(4)* as adapted for vegetables by Moyer and Tressler *(16)*. Riboflavin was determined by the method of Connor and Straub *(5)* as modified by Mackinney and Sugihara *(13)*. Only ascorbic acid analyses were made in the supplementary study.

Statistical Treatment. A series of cookings, consisting of one cooking at each of the four pressures, was made on one day. Each study was repeated five times; the supplementary study was repeated twice. A statistical analysis was made to determine whether the observed differences were significant or merely attributable to experimental variation.

RESULTS AND DISCUSSION

Cooking Times. The slight difference in total cooking times at the four pressures was not of practical importance. As the time to come to the three pressures was increased slightly, the time at the pressure was decreased slightly (Table 1).

Palatability. The average palatability scores for the individual quality factors of the frozen peas cooked at each of the four pressures were "good" to "excellent" (Table 2). As large a difference occurred between the scores of the six judges as between the scores for the peas cooked by the four methods. The differences in scores were not significant.

TABLE 2

Palatability scores for frozen peas cooked at four pressures[a, b]

Cooking pressure, lb. (gauge)	Color score		Texture score		Flavor score		Total score	
	Average	Range	Average	Range	Average	Range	Average	Range
(Atmospheric)	5	4-5	5	4-5	5	3-5	15	11-15
5	5	4-5	4	3-5	4	4-5	13	13-14
10	5	4-5	5	4-5	4	4-5	14	13-15
15	5	None	4	3-5	3	None	14	13-15

[a] Scores of six judges.
[b] The maximum score for each of the three quality factors was five.

Vitamins. The raw frozen peas averaged 18 (17 to 21) mg. of ascorbic acid per 100 g.; 6.3 (1.8 to 12) γ of free folic acid; 18 (8.3 to 24) γ of total folic acid; 0.31 (0.28 to 0.33) mg. of thiamine; and 0.12 (0.11 to 0.14) mg. of riboflavin per 100 grams. The ascorbic acid content of the raw frozen peas used in the supplementary study averaged 16 mg. per 100 grams.

No significant differences in ascorbic acid retention in the frozen peas occurred at the four pressures. Retentions ranged from 63 ± 1.0 to 72 ± 2.2% in the peas after 10 minutes of standing; no water remaining. These retentions are lower than those previously obtained in this laboratory by

Maloney *(15)* using similar cooking methods. In the latter study, however, the peas were analyzed as soon as they were "done."

Retentions of free folic acid ranged from 86 ± 14.6 to 113 ± 9.5%; the differences were not significant. Although some of the folic acid assay methods use heat treatment to liberate the conjugate, the free folic acid content did not increase with the increased temperatures used in this study. Retentions of total folic acid ranged from 81 ± 12.5 to 115 ± 10.6%; differences were not significant.

No significant differences in the percent retentions of thiamine or of riboflavin in the four methods of cooking were observed. The range of retentions were 93 ± 2.2 to 98 ± 2.0% for thiamine; and from 86 ± 5.1 to 92 ± 0.6% for riboflavin as shown in Table 3.

TABLE 3

Retentions of ascorbic acid, free folic acid, total folic acid, thiamine, and riboflavin in frozen peas cooked at four pressures[f]

Cooking pressure, lb. (gauge)	Ascorbic acid	Folic acid		Thiamine	Riboflavin
		Free	Total		
	%	%	%	%	%
(Atmospheric)	63±1.0	86± 7.4	81±12.5	93±2.2	89±4.1
5	69±2.6	113± 9.5	103±10.4	97±1.4	92±0.6
10	72±2.2	106±15.4	115± 7.8	96±2.6	88±2.8
15	68±2.4	86±14.6	115±10.6	98±2.0	86±5.1

[f] Each figure is the mean value of five replications. Four raw samples were taken for each replication.

TABLE 4

The effect of holding after cooking on the retention of ascorbic acid in frozen peas cooked at atmospheric pressure[g]

Time between cooking and sampling	Ascorbic acid retention		
	Peas	Cooking water	Total
min.	%	%	%
0	71 (68-74)	12 (9-14)	83
10	68 (63-73)	....	68 ....

[g] Two replications of each cooking were made; four raw samples were analyzed.

Previous studies have shown that the oxidation of ascorbic acid is correlated with the length of time of exposure to heat and air. A supplementary study was made to test the hypothesis that the low ascorbic acid retentions reported in the body of this study were the result chiefly of the 10-minute holding period.

Total retentions ranged from 77 to 88% in those peas sampled immediately after cooking, as shown in Table 4. These retentions are similar to those reported in the literature.

A 100 g. serving of the frozen peas, cooked by any one of the methods used, provided approximately one-fifth of the 75 mg. of ascorbic acid, about one-fifth of the 1.5 mg. of thiamine, and slightly less than one-tenth of the

1.8 mg. of riboflavin recommended as the daily allowance by the National Research Council. No daily allowance of folic acid has been recommended.

SUMMARY

Commercially frozen peas were cooked in amounts similar to those used in home cooking at the four cooking pressures ranging up to 15 pounds (gauge). Total cooking times, palatability, ascorbic acid, free and total folic acid, thiamine, and riboflavin retentions were determined. An interval of 10 minutes was allowed between cooking and sampling.

Differences in total cooking times were too small to be of importance when the time to bring to boil, to pressure, and at pressure were considered.

No significant differences in palatability scores resulted. The peas cooked by each method were considered "good" to "excellent."

No significant differences occurred in the percent retentions of any of the vitamins in peas cooked at any of the four pressures. The 10 minutes of holding, simulating home treatment, evidently resulted in appreciable loss of ascorbic acid, as shown by a supplementary study.

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THE EFFECT OF SUBTILIN ON THERMOPHILIC FLAT SOUR BACTERIA[a]

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The high heat resistance of the thermophilic spoilage bacteria, and of some *Clostridium* types, makes necessary a heat treatment of low acid canned foods greater than required just to cook to optimum edibility. Consequently there must be more retorts, more steam capacity, more cooling capacity, and perhaps more manpower, for a stated amount of production than would be required if a less severe type of treatment could be used. Any development which would result in increased production, or improved quality, without an enlargement of plant capacity or personnel would be of tremendous commercial significance, and would find ready and widespread adoption by the canning industry. However, any procedure which may be advocated as a substitute or modification for the presently used processing under steam pressure must withstand rigorous scrutiny and practical testing by the industry and its associated technologists. Since spoilage bacteria and bacteria of health significance ordinarily respond similarly to physical and chemical environmental changes, it is common to think first of the effect of a procedural change on the more resistant spoilage types and to evaluate the health hazard only if other considerations suggest or indicate the need for doing so.

The dramatic and spectacular results which follow the therapeutic use of selected antibiotics in certain infectious diseases early stimulated consideration and discussion of the use of antibiotics in other connections in which bacteria are involved. Only a few of these investigations progressed to the point of publication. The selective action of antibiotics has made it obvious that a combination of an antibiotic with some other chemical or physical agency would have to be used in order to obtain complete effectiveness, and in cases where enzyme activity was of importance it was clear that heat would need to be used to a limited degree.

Curran and Evans *(6)* were the first to record the results of the use of an antibiotic in experiments on food preservation. In 1945, they noted that penicillin even in relatively low concentrations would exhibit a sporicidal effect against several aerobic gram positive spore formers, including the thermophilic spoilage type, *Bacillus stearothermophilus*. They noted that the activity was more pronounced in milk than in water, and that sublethal heat treatment before the addition of penicillin usually enhanced the activity. Obviously, though, their results were not provocative of

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extended study of this antibiotic as a food preservative. In the *Annual Report* of the Research Laboratory of The National Canners Association for 1950 *(4)* it is stated that experimentation as early as 1945 indicated that penicillin was not effective with the organisms concerned in the spoilage of canned food.

A great deal of both technical and lay interest in the antibiotic preservation of foods has been stimulated by the report by scientists of the Western Regional Research Laboratory of the United States Department of Agriculture of the successful use of a combination of mild heat with the antibiotic subtilin. Andersen and Michener *(2)* stated that peas, asparagus, corn, green beans, peeled potatoes, tomato juice, and milk had each been protected against spoilage for extended periods of time by a combination of subtilin and a heat treatment less than that customarily used in the canning industry. Some of their containers had been inoculated with *Bacillus stearothermophilus*. The procedure thus would seem to be equally effective with a variety of foods which under natural conditions are subject to spoilage by almost all of the recognized spoilage types, and which are given different processes under the schedules currently in use.

The effectiveness of the antibiotic in media against *B. stearothermophilus*, *Clostridium botulinum*, type A, and a strain of *Bacillus subtilis* is given in this same publication by Andersen and Michener. They state that *B. stearothermophilus* was inhibited in nutrient glucose broth by 0.004 p.p.m. of subtilin, and that *Clostridium botulinum*, 62A, failed to develop in brain-heart infusion within 7 months following 5 minutes of heating at 100°C.(212°F.) in the presence of 5 p.p.m. of subtilin. In a later report *(3)* it was stated that the spores of *B. stearothermophilus* (NCA 1518) and of *B. thermoacidurans* were not greatly affected in buffer even at 100°C. (212°F.), but that in media growth was restrained by less than 0.1 p.p.m. of subtilin. Spores of *Cl. botulinum* heated in water with subtilin for 10 minutes at 88°C.(180°F.) "were destroyed or rendered highly sensitive to subtilin (0.4 p.p.m.)," and were destroyed in pea medium with 10 p.p.m. of subtilin in less than 10 minutes at 100°C.(212°F.).

Studies to confirm and extend the observations of Andersen and Michener have not been encouraging. Tarr, Southcott, and Bissett *(7)* obtained preservative action with fish with aureomycin and terramycin, and slight action with penicillin and streptomycin, but subtilin was ineffective. Investigators at the Washington Laboratory of the National Canners Association *(4)* were unable to confirm the theory of the USDA scientists that spores which survive the subtilin-heat treatment are destroyed by the subtilin as they undergo germination. They state that the effects of the subtilin appeared to be transitory, and that a majority of the organisms grew after 60 days of incubation in concentrations as high as 10 p.p.m. of subtilin. They noted that the thermophilic types are more sensitive than the mesophilic, including *Cl. botulinum*. They obtained spoilage of experimental packs processed for 35 minutes at 100°C.(212°F.) of a variety of foods inoculated with putrefactive anaerobes or with *Cl. botulinum*, both types A and B, with concentrations of subtilin as high as 80 p.p.m. and with spore concentrations as low as 1000 per can. In uninoculated controls with added subtilin spoilage was reduced or lacking. Apparently subtilin

was effective in preventing, or delaying, *thermoacidurans* spoilage of tomato juice, and flat sour spoilage of both corn and peas.

Adams, Ayres, and Tischer *(1)* have found subtilin to be of limited value even in high concentrations (1000 p.p.m.) with comminuted meat inoculated with putrefactive anaerobe No. 3679 and given a mild heat treatment.

The present report is concerned with some of the effects of subtilin in medium on the spores of obligate and facultative thermophilic flat sour bacteria. Apparently within this broad group only strain 1518 of the National Canners Association, a facultative type, has been previously tested.

EXPERIMENTAL METHODS

The organisms included in the work consisted of twenty strains of obligate thermophilic flat sour bacteria and ten strains of facultative types picked at random from the culture collection of the National Canners Association through the courtesy of C. W. Bohrer. The characteristics of each were confirmed before use. It is believed that the number of strains studied gives an adequate representation of the flat sour group, both obligate and facultative types. All of the original isolations were from spoiled canned food. The subtilin was supplied by Merck and Company through the courtesy of H. B. Woodruff. It had a stated potency of 20,000 units per mg.

Spore suspensions were prepared from pure culture isolations of each strain by growing the organism in Kolle flasks on brain-heart infusion at 55°C.(131°F.). About 99% sporulation was obtained within 3 to 5 days. The spores were harvested with normal saline, washed, the suspensions standardized by Petroff-Hauser counts, and stored under refrigeration.

The first experiment was designed to test the effect of subtilin concentration, with and without mild heating, on thermophilic spore germination and growth. Tubes of 1% Albimi C peptone medium, pH 6.8, were prepared with subtilin concentrations of 0, 0.5, 1, 3, 5, 10, 15, 20, 50, and 100 p.p.m. Each tube was seeded with 30,000 spores, which was the same as the inoculum used in the Washington Research Laboratory of the National Canners Association in related work (5). Duplicate tubes were placed under incubation at 55°C.(131°F.) without heat treatment, and duplicate tubes were heated for 5 minutes at 100°C.(212°F.) before incubation. Periodic observation for growth was made. Negative tubes were held for 40 days before being discarded.

EXPERIMENTAL RESULTS

All tubes containing 0, 0.5, and 1 p.p.m. of subtilin showed growth within 72 hours, and all with 50 and 100 p.p.m. were negative through the 40-day incubation period. Two of the facultative thermophiles with 3 p.p.m. of subtilin and four with 5 p.p.m. were delayed in growth for 7 days and one facultative type was restrained by 5 p.p.m. for the full observation period. One heated facultative type did not grow with 5 p.p.m. of subtilin although the unheated tubes showed growth after 7 days. All other tubes with 3 and 5 p.p.m. of subtilin showed growth within 72 hours.

With 10 p.p.m. of subtilin two obligate thermophilic types were inhibited, both in heated and unheated tubes, one was delayed in growth for 7 days in the unheated tube and inhibited in the heated, one was delayed for 7 days in the unheated and 9 days in the heated tubes, and one was delayed for 7 days in both heated and unheated tubes. All others were positive within 72 hours. At 15 p.p.m. all facultative types were inhibited for the 40-day observation period. Six obligate types grew in 72 hours, 3 grew only after 7 days, and two grew after 7 and 9 days for the unheated

and heated tubes, respectively. When the subtilin was increased to 20 p.p.m. only 3 of the obligate types continued to show growth within 72 hours. Two grew after 7 days and one grew in the unheated but not the heated tube. Oddly, one facultative type, which was restrained with 15 p.p.m., showed 7-day growth in the unheated tube. All other tubes remained negative.

Growth of Strain 1518 was delayed until 7 days by 3 and 5 p.p.m. of subtilin, and completely inhibited by 10 or more p.p.m.

These results suggest a slightly greater sensitivity to subtilin on the part of the facultative types. They hardly indicate any effect attributable to the mild heating. Only 4 strains, and distributed among 3 concentrations of subtilin, failed to grow from the heated tubes in parallel with growth from the unheated tubes.

The next experiment was designed to compare quantitatively the amount of growth in the presence and absence of a fixed amount of subtilin of a small group (4 strains each) of obligate and facultative types which had been shown in the first experiment to be of unequal subtilin sensitivity. One of the obligate types showed the development of turbidity with 15 p.p.m. of subtilin but not 20 p.p.m. Three developed turbidity with 20 p.p.m. but not 50. Two of the facultative types developed turbidity with 10 but not 15 p.p.m. of subtilin, one with 5 but not 10 p.p.m., and one with 3 but not 5 p.p.m.

Duplicate tubes of 1% Albimi peptone C broth with and without added subtilin at a level of 10 p.p.m. were inoculated with approximately 30,000 spores per tube, heated for 5 minutes at 100°C.(212°F.) and incubated at 55°C.(131°F.). Quantitative counts by dilution plating were made immediately following heating, and at 1, 3, 7, 14, and 21 days. Maximum growth seemingly was attained by each of the obligate forms at about the same time and with no apparent effect due to the presence of the subtilin. A progressive decline in numbers in each type of culture, which was observed beginning with the 7-day counts, was perhaps a little more pronounced in the absence of subtilin than in the presence. From these results with the limited number of obligate thermophilic types it would appear that a sub-inhibitory amount of subtilin does not delay the attainment of maximum growth, and possibly may favor survival.

With the facultative types the results were more striking and are perhaps best presented in tabular form (Table 1).

These results emphasize that subtilin is of unequal effectiveness with different strains of (presumably) the same organism. They also show that it is not sound to judge the effectiveness of the antibiotic on the appearance of turbidity within a short period of time in a liquid medium. On the basis of the development of turbidity two of the organisms would have been recorded as inhibited after 21 days. By quantitative counts it was established that a slow recovery following an initial sharp decline was in progress. It should be recalled that each of these strains had failed to develop turbidity in broth within 40 days with a lesser amount of subtilin. It is possible that if each organism, both obligate and facultative, had been followed for a longer time similar results would have been recorded.

These data are in harmony with the observation of sporistatic activity noted by the workers at the National Canners Association laboratory.

This experiment was repeated with a spore inoculum of 500,000 per tube and with 50 p.p.m. of subtilin added. All tubes were heated at 100°C. (212°F.) for 5 minutes. In all cases the tubes without subtilin attained maximum growth quickly, followed by a gradual decline through the 21-day count. With added subtilin there was an initial decrease from 200,000 to as low as 12,000 at one day, and the decrease continued to the third day. By the 7-day count most of the subtilin containing tubes were showing recovery, and at the 14- and 21-day counts all showed a continuing increase, but still had not regained the numbers of the original inoculum.

TABLE 1

Effect of subtilin on the growth of facultative flat sour organisms

Strain No. of organism	Subtilin concentrations permitting and restraining turbidity in broth, p.p.m.	Subtilin added, p.p.m.	Counts in thousands per ml. after incubation for days shown					
			0	1	3	7	14	21
1518	5, 10	0	25	2,444	2,320	....[b]	....[b]	....[b]
		10	32	18	11	8	14	21
1863	10, 15	0	30	1,510	1,890	....[b]	....[b]	....[b]
		10	28	930	1,040	....[b]	....[b]	....[b]
1750	3, 5	0	31	2,310	2,260	....[b]	....[b]	....[b]
		10	31	13	6	0.9	3.2	14.6
4135	10, 15	0	26	1,980	2,050	....[b]	....[b]	....[b]
		10	30	40	64	895	....[b]	....[b]

[b] Negative growth phase. Count decreasing.

The results of from 3- to 5-replicate experiments dealing with each point discussed were in close agreement.

These data establish that even in the presence of appreciable amounts of subtilin in broth some cells will survive and growth will take place. They also suggest that subtilin-resistant strains may be developed by the same technique which has been used successfully with other antibiotic and chemotherapeutic agents.

The data here presented suggest that subtilin is not effective in preventing the survival and growth from the spore state of flat sour bacteria.

SUMMARY

A combination of subtilin and mild heat treatment has been found ineffective for the complete destruction of the spores of a number of obligate and facultative thermophilic flat sour bacteria.

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THE EFFECT OF CERTAIN VARIATIONS IN FAT, YEAST, AND LIQUID ON THE FROZEN STORAGE OF YEAST DOUGHS[a]

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The quality of breads made from frozen yeast doughs may be influenced by the various ingredients in the formula. McPherson and Lamb *(7)* reported that the amount of fat, sugar, yeast, or whole wheat flour affected the flavor and texture of bread baked from frozen doughs. These workers found that increasing the fat content of their formula above 3.4%, based on flour, resulted in an unacceptable product at the end of four weeks. Godkin and Cathcart *(5)* in a study of the survival of yeast in frozen doughs used a roll formula containing 12.5% of fat and reported normal yeast activity up to three weeks. Since homemakers would probably use for freezing a standard bread formula such as that given by King and Freeman *(6)* containing 2.5% fat, it would seem that further work is needed on the effect of the level of fat on the quality of bread made from frozen yeast doughs. Compressed yeast has been generally used in studies on the quality of breads prepared from frozen yeast doughs *(5, 7)*. Godkin and Cathcart *(5)* reported that different brands of compressed yeast varied as to their ability to withstand storage at −23.5°C.(−10°F.) over a period of seven months. Although dry yeast is frequently used by homemakers, there is little information in the literature concerning the freezing quality of doughs made with this type of yeast. The addition of milk or milk solids to a formula has been found to modify the properties of unfrozen yeast doughs *(2, 3)*. Previous studies on the frozen storage of yeast doughs *(5, 7)* were made with milk as the liquid in the formula.

The present study was planned to investigate the freezing qualities of yeast doughs prepared from formulas with variations including two levels of fat, 2.5 and 12.5%; two types of yeast, dry and compressed; and two types of liquid, whole fresh milk and water. The doughs were tested after one day, and after one, four, and eight weeks of frozen storage. The one-day and one-week tests were included since previous work in this laboratory on the freezing of baked cakes and cake batters *(9)* showed that when significant changes took place in these experimental products, the change was evident early in the storage period. The study was terminated at the end of eight weeks since the storage of such products for longer periods may not be economical *(4)*.

MATERIALS AND METHODS

A standard bread formula *(6)*, with certain modifications for freezing, was used in this study. Following preliminary work in this laboratory, the standard formula was

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modified by increasing the sugar content and decreasing the yeast content of the standard formula to produce a satisfactory loaf of bread from frozen doughs. These modifications have also been suggested by Fenton and Darfler *(4)* and McPherson and Lamb *(7)*. One lot of each type of yeast was used throughout. Preliminary studies with these lots indicated that 0.5% of dry yeast was equivalent to 1.1% of compressed yeast. The ingredients, including variations in type of yeast, level of fat, and liquid, were as follows:

Ingredients	Amount	Percentage based on flour
Flour, all purpose	2,197 g.	100.0
Sugar	139 g.	6.3
Salt	53 g.	2.4
Yeast:		
Dry	11 g.	0.5
or		
Compressed	24 g.	1.1
Fat, hydrogenated vegetable oil:		
Standard: 2.5%	55 g.	2.5
or		
High: 12.5%	275 g.	12.5
Liquid:		
Milk, whole fresh[b]	1,380 ml.	62.8
or		
Water	1,200 ml.	54.6
Water to soak yeast	200 ml.	9.1

[b] The milk contained 87% water, 3.7% fat, and 9.3% other solids.

The ingredients were obtained from single lots in ample quantity to complete the study. Each batch was sufficient for six one-pound loaves. There were five replications of each treatment. The doughs were mixed by the straight dough method *(6)* using a model C-10 Hobart electric mixer with a ten-quart bowl and dough hook. All procedures were standardized. The formula variations used were as follows: 1, compressed yeast, whole fresh milk, and 2.5% fat; 2, dry yeast, whole fresh milk, and 2.5% fat; 3, dry yeast, water, and 2.5% fat; and 4, dry yeast, whole fresh milk, and 12.5% fat.

Fresh Dough. A 525-g. portion of fresh dough was placed in a small greased bowl and fermented 105 minutes at a temperature of 29°C.(84° ± 2°F.) and a relative humidity of 70% or over. Fermentation and proofing of the doughs were carried out in a thermostatically controlled drying oven which had been modified for use as a proofing oven. After fermentation, the doughs were punched down, molded into loaves following the procedure of the American Association of Cereal Chemists *(1)*, placed in bread pans of a standard size and shape, and allowed to proof until the dough reached the top of the baking pan. All proofing times were recorded. The loaves were then baked at 204.5°C.(400°F.) for thirty-five minutes.

Frozen Dough. The doughs to be placed in frozen storage were divided into 600-g. portions and frozen at once after mixing, without rising, as recommended by other workers *(5, 7)*. Since rapid freezing is desirable *(4, 5)* the portions of dough were pressed into sheets (6 x 6 x 1 inches), packaged in aluminum foil using a drug store wrap, and placed in the cold portion of a commercial home freezer. The frozen packages of dough were placed in lightweight cardboard boxes and stored at −23.5°C.(−10°F.) for periods of from one day to eight weeks.

At the end of each storage period certain of the wrapped packages of dough were thawed in the proofing oven for two hours *(4, 7)*. Each sample was removed from the wrapper, scaled to 525 g. and molded in the same manner as the fresh dough and proofed to one-half inch above the top of the baking pan. This proofing height was greater than that used for the fresh dough. In preliminary work, these conditions were

found optimum for the handling of frozen doughs. All proofing times were recorded. These loaves were baked under the same conditions as the fresh dough.

Testing. Volume determinations by the rape seed displacement method were made the day the bread was baked. Moisture analyses, following the vacuum-oven method outlined in Cereal Laboratory Methods *(1)*, and palatability scores were obtained on the succeeding day. The quality rating sheet used in this study included subjective palatability scores as well as a rating based on the objective volume measurement. The latter factor was included since the volume measurement may be an index of the lightness of the bread. The samples were rated on the basis of a maximum score of 100 points apportioned among the following quality characteristics: volume, 20; external appearance, 8; internal appearance, 34; odor, 4; and eating quality of crust and crumb, 34.

RESULTS AND DISCUSSION

Mean proofing times of molded loaves made from fresh and frozen yeast doughs are given in Table 1. The proofing times of the various fresh doughs include the fermentation time in the bowl (one hour and 45 minutes). This total time of rising (fermentation plus proofing) for the fresh doughs is comparable to the proofing time of the molded loaves

TABLE 1

Mean proofing times of molded loaves of bread made from fresh and frozen yeast doughs

Formula variations	Period of frozen storage				
	0[c]	1 day	1 wk.	4 wk.	8 wk.
	min.	min.	min.	min.	min.
Compressed yeast, milk, 2.5% fat	206	198	203	209	224
Dry yeast, milk, 2.5% fat	195	162	192	187	203
Dry yeast, water, 2.5% fat	200	189	196	201	222
Dry yeast, milk, 12.5% fat	207	168	198	200	225

[c] The proofing times for fresh doughs include fermentation time in bowl (1 hr., 45 min.) plus proofing time of molded loaf. Doughs placed in frozen storage were not allowed to ferment in bowl prior to freezing.

TABLE 2

Mean volumes of loaves of bread made from fresh and frozen yeast doughs

Formula variations	Period of frozen storage				
	0	1 day	1 wk.	4 wk.	8 wk.
	ml.	ml.	ml.	ml.	ml.
Compressed yeast, milk, 2.5% fat	1504	1410[d]	1438	1458	1431
Dry yeast, milk, 2.5% fat	1522	1484	1494	1485	1453
Dry yeast, water, 2.5% fat	1502	1473	1493	1515	1479
Dry yeast, milk, 12.5% fat	1452	1421	1459	1486	1459

[d] Significant at the 1% level. Difference in means necessary for significance: 50 ml. at 1% level, 36 ml. at 5% level.

made from the frozen yeast doughs since the latter were not allowed to ferment in the bowl. In general, the proofing times of the doughs made from the four formula variations, either fresh or frozen from one day to eight weeks, are similar.

Mean volumes of breads made from fresh and frozen yeast doughs are given in Table 2. The data were treated statistically according to the method of analysis of variance *(8)*, and differences in means necessary for

significance were calculated making it possible to determine when significant changes in volume took place. Freezing caused a highly significant decrease in the volume of bread made from frozen, thawed dough containing compressed yeast, milk, and 2.5% fat. The decrease was evident after only one day of frozen storage. A similar finding was reported from this laboratory in a study of frozen cakes and cake batters where significant changes took place early in the storage period *(9)*. Breads made from these compressed yeast doughs, held in frozen storage for one week or longer, had somewhat higher volumes than the breads made from the same type of dough frozen for one day, although they were still significantly lower in volume than those of the fresh controls. This tendency to increase in volume with increasing periods of frozen storage agrees with work reported by McPherson and Lamb *(7)* who found that breads made with older doughs were lighter than breads prepared from dough frozen for two weeks.

Breads made from frozen, thawed dough containing dry yeast, milk, and 2.5% fat showed no significant decrease in volume after eight weeks of frozen storage. A comparison of the two types of yeast shows that after the doughs had been frozen, the volumes of the breads made with the dry yeast, milk, and 2.5% fat were significantly higher than those of the breads made with compressed yeast, milk, and 2.5% fat.

TABLE 3

Mean quality ratings[f] for bread baked from fresh and frozen yeast doughs

Formula variations	Period of frozen storage				
	0	1 day	1 wk.	4 wk.	8 wk.
	%	%	%	%	%
Compressed yeast, milk, 2.5% fat	89	72	77	71	66
Dry yeast, milk, 2.5% fat	93	84	80	76	64
Dry yeast, water, 2.5% fat	88	80	81	82	69
Dry yeast, milk, 12.5% fat	81	68	74	74	62

[f] Based on a maximum score of 100 points.

The volume of the bread made from frozen, thawed dough containing dry yeast, water, and 2.5% fat was not affected by frozen storage and was not significantly different from the volume of the bread made with dry yeast, whole fresh milk, and 2.5% fat.

When the formula contained dry yeast, milk, and 12.5% fat, frozen storage of the dough caused no significant decrease in the volume of the breads. Statistical analyses of the data showed that the volumes after one day of frozen storage were significantly lower than those of the breads made from doughs containing dry yeast, milk, and 2.5% fat. However, at the end of four and eight weeks of frozen storage this difference was no longer apparent.

The moisture content of the breads made with 2.5% fat, compressed or dry yeast, and milk or water were quite similar with a mean of 32.6% for the fresh samples and 33.2% for those from the frozen doughs. The breads made with 12.5% fat in the formula had a somewhat lower mean moisture content, 30.6% when fresh and 31.2% after frozen storage.

Mean quality ratings[*] for bread baked from fresh and frozen yeast doughs are given in Table 3. Ratings of all breads made from frozen dough were considerably lower than those of the fresh controls, and after eight weeks of frozen storage all the samples were of poor quality with scores of 62 to 69 points. Since the quality scores were decidedly lower at eight weeks than at four weeks, it appears that doughs should not be held in frozen storage for this length of time. In an earlier study on the effect of frozen storage upon cakes and cake batters *(9)* the authors reported the occurrence of an undesirable compact layer in many of the products after frozen storage. In the present study there were practically no heavy layers observed in the breads made from fresh doughs. However, after the dough had been in frozen storage only one day these undesirable layers were evident. This tendency was pronounced in bread made from doughs stored for one week and longer, with 80% of the loaves showing this layer. In general, the grain of the bread made from frozen, thawed dough was coarser and less uniform than that of bread made from fresh dough. This agrees with the findings of McPherson and Lamb *(7)*.

Before and after frozen storage the breads made with the dry yeast, milk, and 2.5% fat were of somewhat better quality than those made with the compressed yeast. This superiority of dry yeast over compressed yeast in maintaining the quality of bread from frozen doughs together with the volume measurements, indicate that up to four weeks the dry yeast was more satisfactory than compressed yeast for frozen yeast doughs.

The effects of varying the type of liquid in the formula were inconsistent. Before freezing and after one day of frozen storage the breads made with dry yeast, whole fresh milk, and 2.5% fat were slightly more palatable than breads made from doughs containing water as the liquid. At later test periods this relationship was reversed and the water breads were scored somewhat higher than the milk breads. The total fat content of the breads prepared with milk was greater than that of the breads prepared with water due to the fat content of the milk. However, no consistent differences in proofing time, volume, and palatability were noted between the breads prepared with milk doughs and the breads prepared with water doughs when 2.5% fat (hydrogenated vegetable oil) was added.

When 12.5% fat was used in the formula, the mean quality rating of the fresh bread (81 points) was decidedly lower than that of the fresh bread made from a similar formula with 2.5% fat (93 points). When these doughs were frozen for one day the difference in quality ratings was even greater; the products made with 12.5% fat were decidedly lower than those of breads made with 2.5% fat, 68 and 84 points, respectively. With longer storage of the doughs quality differences were less pronounced and at the end of eight weeks breads made with either level of fat were of poor quality. The texture of the breads made with 12.5% fat became progressively coarser with longer storage, a finding similar to that reported by Godkin and Cathcart *(5)*. Increasing the fat content of the formula to 12.5% resulted in smaller volumes (Table 2) as well as lower quality scores and the formula was not satisfactory for frozen storage.

[*] Based on a maximum score of 100 points.

A yeastlike odor was noted occasionally in the fresh control breads made with 12.5% fat, but was not detected in the fresh control breads made from the other formulas. After one or more weeks of frozen storage, this odor was detected in approximately 20% of all the samples but was most pronounced in the bread made with the 12.5% fat. A "yeasty" flavor was rarely detected until the eighth week when about 17% of the samples had this off-flavor. Again, the "yeasty" flavor was most pronounced in the bread with 12.5% fat. No other off-flavors or odors were noted.

SUMMARY

Formula variations used in this study on the frozen storage of yeast doughs included: (1) compressed yeast, whole fresh milk, and 2.5% fat; (2) dry yeast, whole fresh milk, and 2.5% fat; (3) dry yeast, water, and 2.5% fat; and (4) dry yeast, whole fresh milk, and 12.5% fat. Breads made from the frozen doughs were measured for volume and scored for palatability after one day and one, four, and eight weeks of storage at −23.5°C.(−10°F.).

Quality scores of all breads made from frozen doughs were considerably lower than those of the fresh controls. The grain was coarser and less uniform than that of bread made from fresh dough. An undesirable, heavy compact layer was present in 80% of the loaves after one week of frozen storage and 20% of the samples had a "yeasty" odor. Breads made from doughs frozen four weeks were quite similar in quality to those made from doughs frozen only one day. After eight weeks of frozen storage all samples were of poor quality and storage for this length of time was considered unsatisfactory.

Under the experimental conditions of this study, dry yeast was more satisfactory than compressed yeast for use in the frozen storage of doughs up to four weeks. After the doughs had been frozen the volumes of the breads made with the dry yeast, milk, and 2.5% fat were significantly higher than those of the breads made with compressed yeast. The volumes of the breads made with the compressed yeast were significantly lowered after one day of frozen storage. The volumes of the breads made from formulas containing dry yeast, milk, or water, and 2.5 or 12.5% fat were not significantly affected by eight weeks of frozen storage.

The results of volume and palatability measurements indicated no consistent difference in the quality of the breads made from frozen doughs with either whole fresh milk or water as the liquid in the formula.

Increasing the fat content of the formula from 2.5 to 12.5% resulted in smaller volumes as well as lower quality scores and the formula was not considered satisfactory for frozen storage.

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LIGHT DESTRUCTION OF RIBOFLAVIN IN BAKERY PRODUCTS[a]

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It is widely accepted that riboflavin is thermostable during the ordinary processing of foods but is particularly susceptible to destruction by light. In our examination of bakery products to determine their compliance with standards for enrichment, we have observed deficiencies of riboflavin that could be explained only on the basis of loss on exposure to light. These observations were made on partially baked rolls found to contain less than the labeled amount of riboflavin, where factory inspections indicated that proper manufacturing processes had been followed. We are not familiar with reports dealing specifically with the stability of riboflavin in bakery products during their manufacture and distribution, but studies have been conducted on the vitamin losses in such products with emphasis on the heat destruction of thiamine. Meckel and Anderson *(3)* found no loss of thiamine, riboflavin, and niacin during a two-week storage period of the Army field bread (round loaf). Andrews *(2)* reported that no measurable loss of riboflavin occurred during the baking of bread but that a marked loss occurred in slices unduly exposed to light. We have emphasized that care is necessary to prevent riboflavin loss due to light exposure during the assay process *(1)*, and particularly during preparation of samples, such as enriched bread that must be dried. Ottes and Roberts *(4)* have suggested the use of window screen filters for excluding light of destructive wavelengths from the laboratory in which samples are being examined.

In the study reported here bakery products exposed to light for various periods of time under conditions that might simulate those met with during retail distribution were examined for riboflavin, thiamine, and niacin content. Because published data on the heat stability of riboflavin under various conditions are limited, it was thought that our study of riboflavin stability to heating over a wide range of pH would be of interest.

EXPERIMENTAL

Stability of riboflavin to heat over a range of pH from 0.1 to 13.0 was determined by heating solutions of measured pH containing 50 micrograms of the vitamin per ml. for 60 minutes in an autoclave at 121-123°C. The following reagents were used in preparing these solutions: from pH 0.1 to 3.07, HCl; from pH 2.07 to 9.25, citrate-phosphate buffer; for pH 13.0, NaOH. The riboflavin content after heating was determined by both the A.O.A.C. microbiological and fluorometric methods *(1)*.

The following bakery products were used in studying the effect of exposure to light: (a) sliced enriched pan bread in one-pound loaves, wrapped in heavy wax paper; (b) partially baked enriched rolls in seven-ounce packages, each containing eight rolls, in containers the tops of which were covered with clear uncolored cellophane; and (c) enriched hearth baked (Vienna) bread in one-pound loaves, unwrapped. The

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samples used were obtained from a local commercial bakery and samples of each type of product were from the same dough mix and baking. A sample of enriched flour, 100-g. portions in pint glass jars, was also included.

The effect of exposure to light, similar in intensity and quality to that which might be encountered in ordinary retailing of bakery products, was also of interest to us. To determine the protective effect of wrapping, samples were exposed both wrapped and unwrapped. The unwrapped sliced bread was held firmly together with cord so that the crust of the bread and not the crumb was exposed to light. For purposes of control, portions of samples were dried immediately in the dark and analyzed. Other portions were treated as follows: (a) a portion was stored in complete darkness; (b) another portion placed on a white surface was exposed to the combined artificial light of fluorescent and incandescent lamps of the laboratory (the room used had no outside source of light and the samples were approximately 9 feet from a 160-watt fluorescent light and 12 feet from a 400-watt incandescent light); (c) another portion was placed on a white surfaced bench in a roof green house fitted with clear glass windows at sides and ceiling for exposure to sunlight. During the period of the experiment (in late November and early December) weather conditions were clear and for the first five days there was maximum sunshine. During the sixth and seventh days the sunshine was approximately 75% of maximum. The unit portions of all samples were placed six inches apart from side to side and end to end. Room temperatures during the experiment were 25°C.

The samples of enriched bread and enriched rolls were examined after periods of 1, 2, 3, and 7 days. The samples of enriched hearth baked bread and enriched flour were examined at the beginning and at the end of the seven-day period. The baked products were prepared for analysis in the following manner: after being broken up by hand, they were dried for 4 hours at 60°C. in a forced-draft oven, then allowed to come to air-dry equilibrium during storage for 48 hours in a dark air-conditioned room. Each portion of several loaves or packages was then finely ground and composited. These composites were assayed as indicated in the accompanying tables, for riboflavin using the A.O.A.C. fluorometric method, for thiamine by the A.O.A.C. fluorometric method, and for niacin by the A.O.A.C. microbiological method *(1)*. The stability of these three vitamins during the drying process, in preparation of the sample for assay, was demonstrated by analysis of samples of fresh bread as received, without drying. The results revealed no loss in these constituents during the drying process.

RESULTS

The stability of riboflavin during 60 minutes of heating at 121-123°C. throughout a wide range in pH is shown in Figure 1. It is apparent that the vitamin is completely stable under these conditions of heating from pH 1.0 to 5.4. As the pH of the solution is changed from 6 to 7 the destruction is increased from 20 to 85% and at pH 8 destruction of the riboflavin is nearly complete. Below pH 1.0 there is also a progressive loss of the vitamin with increase in acidity. The stability of the compound is altered markedly within a narrow range of pH, both on the acid and alkaline side of its stable range.

The pan bread showed no loss of riboflavin when stored in the dark or when exposed to artificial light while wrapped, as shown in Table 1. Losses were found to occur when the unwrapped bread was exposed to artificial light for seven days. However, there was no marked change during the first three days under these conditions. The reduction of riboflavin content in the wrapped bread exposed to sunlight was similar to that observed in the unwrapped bread exposed to artificial light. A marked and significant loss occurred during the first day in sunlight in the bread unprotected by wrapping. This loss was increased upon further exposure, with less than two-thirds of the vitamin remaining after three days and less than half remaining after seven days.

TABLE 1

Effect on riboflavin content of exposing enriched bread to light

Treatment	Time in days				
	0	1	2	3	7
	Riboflavin, mg./lb.				
None (as received)	1.03	.....	.....	.....	.....
Protected from light	.....	.....	.....	.....	1.05
Exposed to artificial light					
Wrapped	.....	.....	.....	.....	1.02
Unwrapped	.....	1.05	1.05	1.00	0.87
Exposed to sunlight					
Wrapped	.....	1.03	1.03	0.99	0.93
Unwrapped	.....	0.90	0.74	0.63	0.47

The enriched hearth baked (Vienna) bread showed smaller loss of riboflavin in sunlight and in artificial light than did the pan bread or rolls; nevertheless the loss noted was significant. It amounted to 7.4% in artificial light and 23.5% in sunlight at the end of the seven-day period. It appears that the darker and heavier crust of the Vienna bread offered

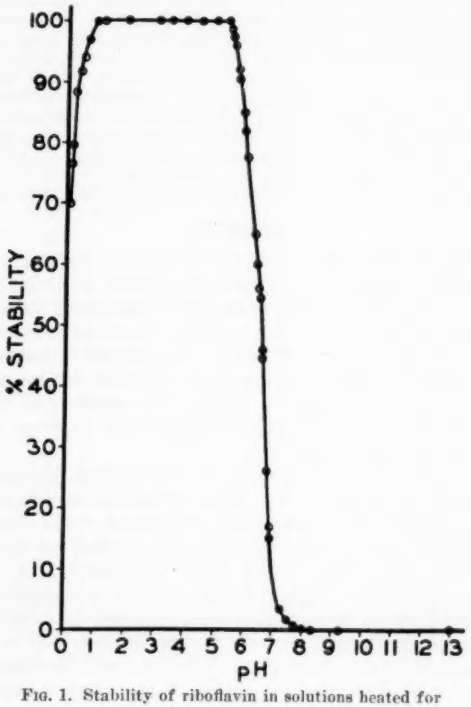

FIG. 1. Stability of riboflavin in solutions heated for 60 minutes at 121-123°C.

greater protection to the riboflavin content. The riboflavin in enriched flour was found to be stable both in artificial light and in sunlight.

The partially baked rolls were found to remain unchanged in riboflavin content over a period of seven days when completely protected from light. In the results presented in Table 2 it will be seen that loss on exposure to artificial light of the cellophane-wrapped rolls was apparent after the second day of exposure. With the wrapping removed the loss was more rapid and occurred to a measurable degree during the first day. In the rolls exposed to sunlight the rate of loss was much greater and in the unwrapped portion the destruction exceeded 75% for the seven-day period. The cellophane wrapping offered slight protection from the effect of sunlight. This product had not been baked for a sufficient period to produce surface color, or any appreciable amount of crust.

TABLE 2

Effect on riboflavin content of exposing enriched rolls to light

Treatment	Time in days				
	0	1	2	3	7
	Riboflavin, mg./lb.				
None (as received)	0.81	.....	.....	.....	.....
Protected from light	.....	.....	.....	.....	0.81
Exposed to artificial light					
Wrapped	.....	0.79	0.74	0.69	0.61
Unwrapped	.....	0.74	0.60	0.54	0.44
Exposed to sunlight					
Wrapped	.....	0.57	0.48	0.43	0.32
Unwrapped	.....	0.42	0.37	0.31	0.19

The stability of thiamine and niacin in these samples is expressed in the results presented in Table 3. There was no measurable loss of these vitamins in any of the products examined.

TABLE 3

Thiamine and niacin content of unwrapped bread and rolls and flour before and after 7 days of exposure to light

Treatment	Bread		Rolls		Enriched flour	
	Thiamine	Niacin	Thiamine	Niacin	Thiamine	Niacin
	mg./lb.		mg./lb.		mg./lb.	
1. None (as received)	1.23	11.0	1.05	9.01	2.00	16.0
2. Exposed to artificial light	1.20	11.0	1.05	9.08	2.02	16.0
3. Exposed to sunlight	1.18	10.7	1.01	9.15	1.96	16.0

DISCUSSION

The results reported here demonstrate the importance of protection of baked products from light that is destructive to riboflavin. The importance, as well, to the analyst who prepares bread samples for riboflavin assay, to avoid exposure to destructive light, should be emphasized. Although those products that are baked to a dark color and heavy crust are of least concern in this respect, some effort is important in providing protective wrapping for pan breads and for the recently marketed partially baked

products. Clear cellophane wrappings offer little protection, either from artificial light or from sunlight.

With respect to stability to light, thiamine and niacin are of no concern. These vitamins were found to be retained in our experiments. The destruction of thiamine by heat in the baking process is well understood, and is accounted for in the standards and labeling of enriched products. Although under relatively drastic conditions of time of heating, and in pure solutions, riboflavin was found to be destroyed at pH 6 to 7, conditions in many foods heat-processed in this pH range serve to protect the vitamin, and manufacturers need not be particularly concerned in this respect with such processing when vitamin retention has been demonstrated. Foods are not ordinarily subjected to extended periods of heat treatment under alkaline conditions because many undesirable changes are produced.

This study serves to emphasize again the need for care in preparation of samples for analysis of their riboflavin content.

SUMMARY

In a study of stability of riboflavin in pure solutions subjected to 60 minutes of heating at 121-123°C., over a wide range of pH, it was shown that the vitamin is completely stable between pH 1.0 and 5.4. With increase in pH, there is a marked loss of riboflavin which becomes complete at about pH 8.0.

The effect on riboflavin content of bakery products exposed to light has been studied. The riboflavin content of pan bread and partially baked rolls, wrapped and unwrapped, was determined before and after such products were exposed to sunlight or to artificial light of the laboratory for periods of 1, 2, 3, and 7 days. Extensive losses were observed in unwrapped rolls with one day of exposure to sunlight and in the unwrapped bread with 3 days of exposure. In addition, there was an extensive loss in the partially baked rolls when exposed, unwrapped, to artificial light and when exposed, wrapped, to sunlight. Translucent wax paper wrapping of bread offered marked protection, but clear cellophane wrapping commonly used for partially baked rolls was of little value in preventing riboflavin loss.

Acknowledgment

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